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**(54) Title:** A METHOD OF IDENTIFYING CFTR-BINDING COMPOUNDS USEFUL FOR ACTIVATING CHLORIDE CONDUCTANCE IN ANIMAL CELLS

**(57) Abstract**

The present invention provides a method of identifying CFTR-binding compounds for treating cells having a reduced apical Cl<sup>-</sup> conductance, such as cystic fibrosis cells. This identification method involves the use of polypeptide I<sup>α</sup>, which constitutes a portion of the CFTR protein. The present invention also provides a method of treating CF cells by contacting cells having a reduced apical Cl<sup>-</sup> conductance with a therapeutically effective quantity of a compound selected by the present inventive identification method. Preferred compounds for such treatment have little or no affinity for adenosine cell receptors. The present invention provides novel compounds useful in practicing the present inventive method, as well as pharmaceutical compositions containing such compounds.

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A METHOD OF IDENTIFYING CFTR-BINDING  
COMPOUNDS USEFUL FOR ACTIVATING  
CHLORIDE CONDUCTANCE IN ANIMAL CELLS

Field of the Invention

The present invention relates to a method of identifying CFTR-binding compounds for treating cells having a reduced apical  $\text{Cl}^-$  conductance, such as cystic fibrosis cells. The present invention further relates to compounds and pharmaceutical compositions thereof useful in treating such cells.

Background of the Invention

Healthy animal cells require, among other conditions and materials, the movement of various inorganic ions across the cell membrane to be maintained such that a proper balance of the ions provide the requisite electrical potential across the cellular membrane as well as a life-promoting internal ionic strength. For example,  $\text{Na}^+$ ,  $\text{Cl}^-$ ,  $\text{K}^+$ , and  $\text{Ca}^{++}$  are known to cross cell membranes in animals such that  $\text{K}^+$  and  $\text{Ca}^{++}$  are accumulated intracellularly to a varying extent in different cells at different times of development, whereas  $\text{Na}^+$ , in large measure, is excluded from the interior of a cell. The cross-cellular movement of these ions is mediated by  $\text{Na}^+/\text{K}^+$ - and  $\text{Ca}^{++}$ -dependent ATPases that are membrane bound at the sites of appropriate ion channels. Chloride ion was believed to permeate animal cells by passive means to equilibrate in concentration between the external and internal fluid, resulting in an underrepresentation of  $\text{Cl}^-$  intracellularly in consequence of the overall negative intracellular charge. Conductance of chloride, however, has been shown to be mediated actively as well, by means of a  $\text{Cl}^-$  channel (see Edwards, Neuroscience, 7, 1335-1366 (1982)).

Results from research directed to the pathology of cystic fibrosis ("CF") has provided information on the ill-effects that an ion conductance impairment at the

cellular level can have on a person's health, at many levels. CF was known to have a genetic basis because of its differential incidence among white Americans (between 1/1600 and 1/2000 live births) as compared to African 5 Americans (about 1/17,000 live births). Indeed, research over the past decade has revealed that a heritable discrete gene mutation is associated with the clinical symptoms of CF, including abnormal exocrine gland and lung functions.

10 More specifically, CF is caused by mutations in the cystic fibrosis transmembrane regulator (CFTR) gene, the most common of which is the deletion of a phenylalanine residue at position 508 (Phe<sup>508</sup>; see Figure 1 for a physical map of the CFTR protein). The mutated CFTR 15 protein is referred to as  $\Delta$ F508, the site of which is within the first nucleotide binding fold (NBF-1).

Schoumacher et al., Proc. Natl. Acad. Sci., 87, 4012-4016 (1990); Riordan et al., Science, 245, 1066-1073 (1979). More specifically, this mutation is located in a lengthy 20 internal segment of the NBF-1, flanked on the N-terminal side by the Walker A sequence (amino acid position 458-471; also referred to as "CFTR[458-471]") and on the C-terminal side by the contiguous C domain (amino acid position 548-560; also referred to as "CFTR[548-560]") and the Walker B domain (amino acid position 561-573; also referred to as "CFTR[561-573]"). The physiological 25 role of the intervening sequence located between CFTR[471] and CFTR[561], which includes the polypeptide sequences of I $\alpha$ , I $\beta$ , and I $\gamma$  (see Figure 1), is unknown.

30 Certain mutations in the CFTR gene, such as that resulting in  $\Delta$ F508, cause an abnormal potential difference across CF epithelia. The abnormality is due to a reduced cellular apical chloride (Cl<sup>-</sup>) conductance. Consequently, chloride and sodium transport across 35 epithelial membranes of an individual afflicted with CF, for example, is abnormal. It is also known that cells carrying the  $\Delta$ F508 mutation have higher than normal CFTR

protein bound to their endoplasmic reticulum (hereinafter, "ER"), although wild type cells also retain and degrade a substantial amount of CFTR protein in their ER, albeit much less so than the  $\Delta F508$  mutant. Ward et 5 al., J. Gen. Physiol., 104, 33a (1994).

This CFTR mutation apparently is responsible for pathophysiological changes in the respiratory system, among others. Nearly all patients suffering from the disease develop chronic progressive disease of the 10 respiratory system. In the majority of cases, pancreatic dysfunction occurs, and hepatobiliary and genitourinary diseases are also frequent.

Although survival of cystic fibrosis patients has improved in recent years, the median survival is still 15 only about 28 years despite the development and implementation of intensive supportive and prophylactic treatment. Present efforts to combat the disease have focused on drugs that are capable of either activating the mutant CFTR gene product or otherwise causing 20 additional secretion of  $Cl^-$  from affected cells. Gene therapy is another area of active research, wherein the anion conductance deficit may be repaired by the introduction of a recombinant wild-type CFTR gene, i.e., a CFTR gene that lacks a mutation that results in the 25 abnormality.

Encouraging clinical results have been reported recently for the use of aerosols containing either amiloride (Knowles et al., N. Engl. J. Med., 322, 1189-30 1194 (1990)) or a mixture of ATP and UTP (Knowles et al., N. Engl. J. Med., 325, 533-538 (1991)), which slow the accumulation of  $Cl^-$  in the epithelium of the trachea.

Other drugs that purportedly are useful in the treatment of CF have been described. For example, U.S. Patent No. 4,866,072 describes the use of 9-ethyl-6,9-35 dihydro-4,6-dioxo-10-propyl-4H-pyrano(3,2-g)quinoline-2,8-dicarboxylic acid or a pharmaceutically acceptable derivative thereof in the treatment of CF. U.S. Patent

No. 4,548,818 describes the use of a 3-alkylxanthine to treat chronic obstructive pulmonary disease (COPD). U.S. Patent No. 5,032,593 describes the use of a 1,3-alkyl substituted 8-phenylxanthine or a pharmaceutically acceptable salt thereof in the treatment of bronchoconstriction. U.S. Patent No. 5,096,916 describes the use of an imidazoline  $\alpha$ -adrenergic blocking agent and vasodilator, such as tolazoline, in the treatment of COPD, including cystic fibrosis, chronic bronchitis and emphysema, or COPD in association with asthma.

Historically, theophylline has been administered to asthmatic and CF patients to enhance lung function. Such lung function enhancement is caused principally by bronchodilation, which is due to the action of theophylline on smooth muscles and inflammation. Theophylline has been shown not only to inhibit phosphodiesterase, but also to antagonize adenosine receptors. Accordingly, because theophylline acts at more than one site, it obviously lacks specificity, thus reducing its usefulness to treat CF. In view of the fact that antagonism of the A<sub>1</sub> adenosine receptor, not inhibition of phosphodiesterase, has been shown to result in stimulating chloride efflux from CF cells, such lack of specificity could result in undesired side effects, such as detrimental effects to cardiac, renal, and/or central nervous system tissue. In addition, large doses of theophylline must be administered to achieve a beneficial effect, thus increasing the risk of side effects from the high toxicity of the compound.

Other compounds that resemble theophylline in basic structure have been tested but have not been found to be useful in evoking chloride efflux from CF cells and, therefore, has no or little potential in the treatment of cystic fibrosis. For example, 3-isobutylmethyl xanthine (IBMX), which is structurally similar to theophylline, is nonspecific in activity and highly toxic and, therefore, lacks utility in the treatment of CF. Also ineffective

in the activation of chloride efflux are the compounds 2-thio-8-cyclopentyl-1,3-dipropylxanthine (2-thio-CPX), 1,3-dipropyl-8-noradamantylxanthine (KW-3902), and 1,3-dimethyl-8-cyclopentylxanthine (CPT) (see U.S. Patent 5,366,977). Similarly, substitution of the propyl group at position 1 or 3 of CPX (1,3-dipropyl-8-cyclopentylxanthine) with a one-carbon group generates a compound that is ineffective in activating chloride efflux from CF cells. Clearly, minor structural differences have a significant, if not substantial, impact on the effectiveness of a given compound in the treatment of CF. Accordingly, it will be particularly useful if a method were available for screening compounds for an ability to promote chloride ion conductance in affected cells, which, in turn, would be a candidate therapeutic agent for treatment of cystic fibrosis patients.

The '977 patent, cited above, and Eidelman et al., Proc. Natl. Acad. Sci. USA, 89, 5562-5566 (1992), disclosed that CPX is a potent A<sub>1</sub> adenosine antagonist that promotes chloride efflux from a human epithelial cell line (CFPAC-1). The CFPAC-1 line, further described in Example 6, expresses the aforementioned CFTR ΔF508 mutation and can be viewed as a first generation screening material for CF-active compounds. Although CPX and its related xanthine amino congeners disclosed in the '977 patent have been shown to be relatively non-toxic and therefore potentially useful for CF treatment, the fact that such compounds have an antagonistic effect on A<sub>1</sub>-adenosine receptors indicates that such treatment probably will have an additional impact on an animal that is unrelated to the CF affliction. Accordingly, use of such compounds, indeed any compound known to have multiple targets of activity, preferably is avoided because the use of such compounds is, at least, potentially detrimental.

A drug of high potency, low toxicity, and little or no specificity for adenosine receptors, thus, would be a highly desirable and promising therapeutic agent for the treatment of cells having a reduced apical  $\text{Cl}^-$  conductance, such as cystic fibrosis cells. Such a drug would not only find utility in the treatment of cystic fibrosis per se but would be therapeutically useful in the treatment of COPD in general.

It is an object of the present invention to provide a method for identifying compounds that can activate an impaired chloride conductance channel. It is also an object of the present invention to provide a method of correcting the reduced  $\text{Cl}^-$  conductance of cells that are impaired in such conductance. It is another object of the present invention to provide a method of treating cystic fibrosis cells. It is yet another object of the present invention to provide a method of treating cystic fibrosis cells having a deletion involving phenylalanine at amino acid position 508 of the cystic fibrosis transmembrane regulator. It is also an object of the present invention to provide compounds useful in such methods, in particular novel xanthine derivatives that have little or no affinity for the adenosine receptors but have an ability nonetheless to ameliorate the  $\text{Cl}^-$  imbalance of CF cells by stimulating  $\text{Cl}^-$  efflux.

These and other objects and advantages of the present invention, as well as additional inventive features, will be apparent from the description of the invention provided herein.

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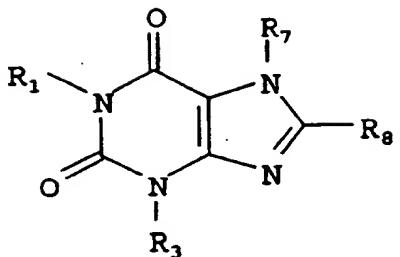
#### Brief Summary Of The Invention

The present invention provides certain CFTR polypeptides, such as I $\alpha$  [SEQ ID NO:1], that exhibit an ability to bind specifically to compounds that are known to activate the CFTR-linked chloride ion efflux in  $\Delta\text{F508}$  mutant cells and not to bind to compounds that are known not to have such a capability to activate CFTR-linked

chloride ion efflux. Moreover, the present invention provides a method for the identification of further compounds capable of activating the CFTR-linked chloride ion efflux. Such a method includes the application of 5 the aforementioned CFTR polypeptides as selective binding agents for ion efflux activating compounds. As a further refinement of the identification method, other CFTR polypeptides, such as  $I\beta$  [SEQ ID NO:3] or  $I\alpha$  [SEQ ID NO:4], having amino acid sequences of adjacent or 10 overlapping regions of the location of  $I\alpha$ , are also disclosed that, despite their close or overlapping proximity to  $I\alpha$ , have a neutral or negative affinity for compounds known to activate ion efflux.

Application of the aforementioned identification 15 method may be used to identify compounds that have an ability to activate ion efflux in ion efflux deficient cells, such as CF cells, especially those having the  $\Delta F508$  mutation. Moreover, such compounds, in the context of the present invention, may be further screened to have 20 little or no affinity for either the  $A_1$ -adenosine cell receptor or the  $A_2$ -adenosine cell receptor. Such compounds, accordingly, may be used in a method of treating cells having a reduced apical  $Cl^-$  conductance, such as cystic fibrosis cells. Specifically, the present 25 inventive method involves contacting cells having a reduced apical  $Cl^-$  conductance with a therapeutically effective quantity of a compound that has little or no affinity for the  $A_1$ -,  $A_2$ -, or  $A_3$ -adenosine cell receptor. Nevertheless, contact between the inventive compounds and 30 CF cells results in  $Cl^-$  efflux from such cells.

In particular, the method involves contacting such cells with a compound having the formula:



wherein R<sub>1</sub> and R<sub>3</sub> are the same and are C<sub>1</sub>-C<sub>6</sub> alkyl or C<sub>1</sub>-C<sub>6</sub> alkenyl, R<sub>7</sub> is C<sub>1</sub>-C<sub>6</sub> alkyl or hydrogen, and R<sub>8</sub> is C<sub>4</sub>-C<sub>8</sub> cycloalkyl. Such a compound may further be selected from the group consisting of 1,3-dipropyl-7-methyl-8-cyclopentylxanthine, 1,3-dipropyl-7-methyl-8-cyclohexylxanthine, cyclohexyl caffeine, 1,3-diallyl-8-cyclohexylxanthine, and therapeutically effective derivatives thereof.

The compounds used in the present inventive method resemble theophylline in basic structure; however, they differ significantly in the substituents at the R<sub>1</sub>, R<sub>3</sub>, and R<sub>8</sub> positions. Given that minor structural differences in compounds that resemble theophylline have been shown to render the compounds ineffective or otherwise not useful in the treatment of CF, it was surprising to discover that the aforementioned compounds are effective in activating chloride efflux from CF cells. This discovery was particularly unexpected inasmuch as the apparent mode of activity of the inventive compounds does not involve any of the adenosine receptors.

25

Brief Description of the Drawings

Figure 1 is a physical map of a portion of the CFTR protein, displaying the location of the following sequences: Walker A (amino acids 458 to 471); I $\alpha$  (amino acids 477 to 508); I $\beta$  (amino acids 509 to 539); I $\alpha$  (amino acids 550 to 530); C (amino acids 548 to 560); and Walker B (amino acids 561 to 573).

Figure 2 is a graph that depicts the aggregation of chromaffin granules by certain CFTR polypeptides at a concentration of 20  $\mu\text{g}/\text{ml}$  (5.2  $\mu\text{M}$ ), at pH 6.0 and 1 mM  $\text{Ca}^{++}$ . The units of the x-axis represent minutes of time 5 and the units of the y-axis represent change in turbidity ( $\Delta 540 \text{ nM}$ ).

Figure 3A is a graph that depicts the aggregation of chromaffin granules in the presence of varying concentrations of the  $\text{I}\alpha$  polypeptide. The units of the 10 x-axis represent the concentration of  $\text{I}\alpha$  polypeptide ( $\mu\text{g}/\text{ml}$ ) and the units of the y-axis represent change in turbidity ( $\Delta 540 \text{ nM}$ ). Reactions were performed as for generating the data of Figure 2; the initial rate was calculated for each concentration of  $\text{I}\alpha$ .

15 Figure 3B is a Hill plot for the data displayed in Figure 3A. The  $V_{\text{max app}}$  was estimated from the linear extrapolation of the Lineweaver-Burk plot ( $\log (V_i/V_m - V_i)$  versus  $\log [\text{I}\alpha]$ ) of the data, and the ratio of velocities was computed for each condition. The slope (2.6) is the 20 Hill coefficient ( $n_H$ ).

Figure 4A is a graph that depicts phosphatidylserine liposome aggregation (y-axis is  $\Delta A_{350}/\text{min}$ ) by  $\text{I}\alpha$  at different pH values (x-axis). The concentration of  $\text{I}\alpha$  is 2  $\mu\text{g}/\text{ml}$  (0.6  $\mu\text{M}$ ).

25 Figure 4B is a graph that depicts phosphatidylserine liposome aggregation (y-axis is  $\Delta A_{350}/\text{min}$ ) by lower concentrations of  $\text{I}\alpha$  polypeptide measured over time (x-axis in minutes).

30 Figure 5 is a graph that depicts the effect of CPX (x-axis in nM) on aggregation of phosphatidylserine liposomes driven by  $\text{I}\alpha$  polypeptide (y-axis is  $\Delta A_{350}/20 \text{ min}$ ). The inset graph depicts the concentration dependence for CPX activation at the 20 minute time point, wherein the y-axis is  $\Delta A_{350}/20 \text{ min}$  and the x-axis 35 is concentration of CPX in nM.

Figure 6 is a graph that depicts the effect of caffeine (x-axis is concentration in nM) on aggregation

of phosphatidylserine liposomes driven by I $\alpha$  polypeptide (measured on the y-axis as  $\Delta A_{350}/20$  min). The inset graph depicts the concentration dependence for CPX activation at the 20 minute time point, wherein the y-axis is 5  $\Delta A_{350}/20$  min and the x-axis is in nM concentration units of caffeine.

Figure 7 is a graph depicting the effect of DAX concentration (x-axis is nM) on aggregation of phosphatidylserine liposomes (y-axis,  $\Delta A_{350}/20$  min) driven 10 by I $\alpha$  polypeptide. The inset graph depicts concentration dependence for DAX activation at the 20 minute time point, wherein the x-axis is nM units of concentration of DAX and the y-axis is in units of  $\Delta A_{350}/20$  min.

Figure 8 is a graph that depicts the comparison of 15 CPX and DAX activation of phosphatidylserine liposome aggregation by I $\alpha$  polypeptide. The x-axis is in nM concentration units of CPX or DAX and the y-axis is in units of  $\Delta A_{350}/20$  min.

Figure 9 depicts a graph of the results of  $[^{36}\text{Cl}]$ - 20 efflux studies of CFPAC cells when placed in contact with compound 1 (theophylline). The x-axis represents the nanomolar concentration of compound 1, and the y-axis represents the rate of chloride efflux as a percentage of control.

Figure 10 depicts a graph of the results of  $[^{36}\text{Cl}]$ - 25 efflux studies of CFPAC cells when placed in contact with compound 5 (CPX). The x-axis represents the nanomolar concentration of compound 5, and the y-axis represents the rate of chloride efflux as a percentage of control.

Figure 11 depicts a graph of the results of  $[^{36}\text{Cl}]$ - 30 efflux studies of CFPAC cells when placed in contact with compound 22 (8-cyclohexylmethyl caffeine). The x-axis represents the nanomolar concentration of compound 22, and the y-axis represents the rate of chloride efflux as 35 a percentage of control.

Figure 12 depicts a graph of the results of  $[^{36}\text{Cl}]$ - efflux studies of CFPAC cells when placed in contact with

compound 17 (1,3-diallyl-8-cyclohexylxanthine). The x-axis represents the nanomolar concentration of compound 17, and the y-axis represents the rate of chloride efflux as a percentage of control.

5 Figure 13 depicts a graph of the result of chloride efflux studies where varying the size of the 8-cycloalkyl substituent (x-axis) of a theophylline derivative is tested for its impact on  $K_i$  value ( $\mu M$ , y-axis). Data of the affinity of 8-cycloalkyltheophylline derivatives on 10 rat cortical  $A_1$ -adenosine receptors (●) and rat striated  $A_{2a}$ -adenosine receptors ( $\Delta$ ) are shown.

15 Figure 14 depicts a graph of the result of chloride efflux studies where varying the size of the 8-cycloalkyl substituent (x-axis) of a caffeine derivative is tested for its impact on  $K_i$  value ( $\mu M$ , y-axis). Data of the affinity of 8-cycloalkyl caffeine derivatives on rat cortical  $A_1$ -adenosine receptors (●) and rat striated  $A_{2a}$ -adenosine receptors ( $\Delta$ ) are shown.

20 Detailed Description of the Preferred Embodiments

The present invention provides polypeptides that have a positive affinity for compounds that activate chloride ion efflux in CFTR-mutated cells. The polypeptides of the present invention are either a 25 portion of the CFTR protein, namely the segment identified in Figure 1 as  $I\alpha$ , or a variant thereof having no significant difference in its functioning, as disclosed herein.  $I\alpha$  is defined as the polypeptide that spans from amino acid position 477 to amino acid position 30 508, and has the following sequence:

NH<sub>2</sub>-PSEGKIKHSGRISFCSQFSWIMPGTIKENEEL-COOH [SEQ ID NO:1]

35 wherein the capital letters between the NH<sub>2</sub> and COOH groups are the conventional single letter symbols for various amino acids, which protein-related nomenclature

is used hereinbelow for SEQ ID NO:3 and SEQ ID NO:4 as well.

Polypeptide I $\alpha$  [SEQ ID NO:1] may be prepared by (1) analytical or preparative polypeptide synthesis using 5 methods well-known in the art (see, e.g., Atherton et al., Solid Phase Polypeptide Synthesis, A Practical Approach (Oxford Univ. Press, 1989)), (2) by appropriate incubation of a microorganism containing a suitable sequence of DNA or RNA for expression of polypeptide I $\alpha$  10 using methods of genetic engineering that are well-known in the art (see, e.g., Sambrook et al., Molecular Cloning: A Laboratory Manual, (2d ed. 1989)), or (3) by any other suitable means. It is contemplated within the context of the present invention that conservative amino 15 acid changes that, as understood within the art, neither present charge nor conformational changes to the polypeptide, constitute further embodiments of the present invention. For example, it is believed that the exchange of an isoleucine for a leucine residue would 20 have little or no effect on the functionality of the polypeptide I $\alpha$ . It is also contemplated that fragments of I $\alpha$  may be functional as well, and thus such fragments define further embodiments of the present invention.

Suitable sequences of DNA and/or RNA for 25 constructing a cloning approach to synthesis of I $\alpha$  may be identified by reverse translation and transcription of the amino acid sequence of I $\alpha$  [SEQ ID NO:1], using the genetic code. Either or both of the DNA strands, i.e., the "sense" and "anti-sense" strands, may be usefully 30 employed in the genetic engineering of clones for synthesis of I $\alpha$ . Similarly, the RNA sequence may constitute the "sense" or "anti-sense" strands, as well. Accordingly, the present invention may be viewed as embodied in the RNA or DNA sequences recited and/or 35 referred to herein.

Any suitable means may be applied for generating the RNA molecules having any of the following sequences:

CCN UCN GAR GGN AAR AUH AAR CAY UCN GGN CGN AUH UCN UUY  
UGY UCN CAR UUY UCN UGG AUH AUG CCN GGN ACN AUH AAR GAR  
AAY GAR GAR UUY

[SEQ ID NO:2]

5 wherein A is adenine, G is guanine, U is uracil, C is cytosine, W is A or U, Y is C or U, R is A or G, H is A, C or U, and N is G, A, C or U, and the symbols are organized in triplets to reflect the codons as translated in an organism. This RNA-related nomenclature is used  
10 hereinbelow for SEQ ID NO:5 and SEQ ID NO:6 as well.

Other RNA sequences that, upon translation, provide the same polypeptide sequence or its functional equivalent are contemplated hereby as well. For example, the triplet UCN can be replaced by the triplet AGY and  
15 the triplet CGN can be replaced by the triplet AGR, and yet result in no change in the resultant polypeptide sequence. In addition to the neutral sequence differences identified at the RNA level, as provided herein, nucleotide changes that provide conservative  
20 changes to the resultant amino acid sequence, as discussed above, are contemplated as well. Thus, for example, the triplet AUH, which encodes the amino acid isoleucine, may be replaced by the triplet CUN, which encodes the isomeric amino acid leucine, without  
25 significant effect on the functioning of the claimed polypeptide. Any of such sequences may be ligated into a suitable nucleic acid vector for propagation and expression in a suitable cellular or viral host, using methods well-known in the art.

30 The genetic complement of the aforeslated RNA molecules, i.e., the anti-sense RNA, and the DNA counterparts to the sense and anti-sense RNA molecules are contemplated as embodiments of the present invention. Suitable means for generating the aforementioned  
35 sequences, their complements, or their DNA counterparts, as well as those disclosed hereinbelow, include in vitro polynucleotide synthesis of a manual or automated nature.

Once constructed, such a sequence may be ligated to other nucleic acid sequences that specify signals for transcription or translation, and to yet other nucleic acid sequences useful as a vector for propagation and/or 5 expression in bacterial, viral, or animal cells. Methods for preparing such nucleic acid constructs are well-known in the art. See Sambrook et al., supra.

The polypeptide or a suitable fragment thereof may be used per se or it may be associated with another 10 compound, such as one or more amino acids, which may be covalently linked together as a second polypeptide, a proteoglycan, a proteolipid, a carbohydrate, or some other suitable macromolecule. Such associations of the inventive polypeptide with another macromolecule may be 15 stabilized by means of covalent bonding, hydrogen bonding, adsorption, absorption, metallic bonding, van der Waals forces, ionic bonding, or other suitable inter- or intramolecular bonds or forces, or any combination thereof. For example, polypeptide I $\alpha$  may effectively be 20 enlarged by the covalent addition of one or more amino acids to the carboxy or amino terminus, or both termini, so long as its essential characteristic of binding to activating molecules for chloride ion efflux in CFTR-mutated cells is retained. A preferred form of the 25 inventive polypeptide comprises the covalent linkage of the polypeptide to a suitable affinity chromatography matrix, such as cellulose, including m-aminobenzylloxymethylcellulose, bromoacetylcellulose, carboxymethylcellulose hydrazide, and cellulose cyclic-carbonate; 6-aminohexanoic acid; sepharose, including sepharose 4B, sepharose 6MB, sepharose 6B, and thiol-sepharose 4B; beads composed of hydrophilic acrylic, polyacrylamide, and agarose; and linear polyacrylamide. 30

Suitable reactive moieties, such as hydrazides, 35 carbonates, and the like, and suitable activators, such as cyanogen bromide, carbonyldiimidazole, chloroformates, and the like, may be applied to the aforementioned

affinity chromatography matrices, using methods and materials well-known to the art, as provided, for example, by the 1993 Sigma Chemical Company catalog at pages 1612-1618 and other sources.

Properties of the I $\alpha$  polypeptide include (1) 5 commencing or causing the aggregation of bovine chromaffin granules in the presence of about 1 mM CaCl<sub>2</sub>; having an apparent  $k_{1/2}$  of 22.2  $\mu$ g/ml or 6.3  $\mu$ M (see Figures 3A and 3B); (2) having activity in a pH range of 10 from about 5.5 to about 7.5, wherein 50% activity was measured at about 6.75 (see Figure 4A); (3) having a synergistic effect on commencing or causing the aggregation of liposomes when combined with a compound that activates chloride ion efflux in cells having the 15  $\Delta$ F508 mutation, which compounds include CPX, caffeine, and N,N-diallylcyclohexylxanthine (DAX) (see Figures 5, 6, and 7, respectively); (4) having no increased effect on commencing or causing the aggregation of liposomes when combined with a compound that is known not to 20 activate chloride ion efflux in cells having the  $\Delta$ F508 mutation, which compounds include 2-thio-CPX and isobutylmethylxanthine (IBMX), among others; (5) having a biphasic interaction with adenosine wherein at lower adenosine concentrations of 70 and 100 nM there was a 25 modest increase in the aforementioned liposome aggregation reaction driven by polypeptide I $\alpha$ , at higher adenosine concentrations such increase in activity was not seen, and at 1  $\mu$ M and greater the aggregation activity was suppressed; and (6) having an apparent  $K_D$  of 30 69 $\pm$ 27 nM with respect to CPX, a xanthine derivative known to activate chloride ion efflux from  $\Delta$ F508 cells.

The present invention also provides certain other CTR polypeptides that are contiguous to polypeptide I $\alpha$  but, nevertheless, have characteristics that are 35 significantly different. Polypeptide I $\beta$  is a sequence of amino acids included in the CTR protein from position 509 to position 539, and has the following sequence:

NH<sub>2</sub>-GVSYDEYRYRSVIKACQLEEDISKFAEKDNI-COOH

[SEQ ID NO:3]

5 Polypeptide Io is another sequence of amino acids included in the CFTR protein at from position 500 to position 530, and has the following sequence:

NH<sub>2</sub>-GTIKENEEFGVSYDEYRYRSVIKACQLEEDIS-COOH

[SEQ ID NO:4]

These polypeptides are contiguous or partially overlap

10 polypeptide I $\alpha$ , yet neither of these polypeptides drives the aggregation of chromaffin granules or synthetic liposomes, nor has any effect on the activity of polypeptide I $\alpha$  as disclosed herein or chloride ion efflux-activating xanthine derivatives. Analogously to 15 the discussion above relating to conservative amino acid changes of I $\alpha$ , it is contemplated that polypeptides having such conservative changes are further embodiments of the present invention.

20 The I $\beta$  and Io polypeptides may be prepared and used as free polypeptides, as fragments thereof, or associated with another macromolecule, as discussed hereinabove for the I $\alpha$  polypeptide. The I $\beta$  and Io polypeptides may be used in affinity chromatography, and used as a negative control relative to the I $\alpha$  polypeptide.

25 As with the I $\alpha$  polypeptide discussed above, suitable sequences of RNA and/or DNA corresponding to I $\beta$  and Io may be identified by reverse translation/transcription of the amino acid sequences of the respective polypeptide, using the genetic code. Accordingly, any suitable means 30 may be applied for generating the RNA molecules having any of the following sequences:

I $\beta$ :

35 GGN GUN UCN UAY GAY GAR UAY CGN UAY CGN UCN GUN AUH AAR  
GCN UGY CAR UUR GAR GAR GAY AUH UCN AAR UUY GCN GAR AAR  
GAY AAY AUH

[SEQ ID NO:5]

Io:

GGN ACN AUH AAR GAR AAY GAR GAR UUY GGN GUN UCN UAY GAY  
GAR UAY CGN UAY CGN UCN GUN AUH AAR GCN UGY CAR UUR GAR  
GAR GAY AUH UCN

[SEQ ID NO:6]

5

Analogously to the I $\alpha$  sequence, other sequences that encode the same or functionally equivalent sequences as compared to SEQ ID NO:5 and SEQ ID NO:6 are contemplated as well. For example, the triplets UCN, GCN, and UUR may 10 be replaced by AGY, AGR, and CUN, respectively, without any resultant amino acid sequence differences. In addition, nucleotide sequences that result in neutral amino acid charges, as discussed above with respect to the I $\alpha$  sequences, are contemplated as additional 15 embodiments of the present invention. Moreover, as with the I $\alpha$  sequence, the RNA complement of SEQ ID NO:5 and SEQ ID NO:6, as well as the DNA counterpart sequences predicated thereon, are included as embodiments of the present invention, and may be determined by the 20 application of the conventional genetic code by an ordinary artisan.

The aforementioned I $\alpha$ , I $\beta$  and Io polypeptides or their functional equivalents may be used in a method for identifying a CFTR-binding compound. Such a method 25 comprises contacting a putative CFTR-binding compound to polypeptide I $\alpha$  [SEQ ID NO:1] under conditions sufficient to allow for binding of the putative CFTR-binding compound to said polypeptide I $\alpha$ , and determining, by any suitable means, whether such binding occurred, which 30 would be indicative that the putative CFTR-binding compound was indeed a CFTR-binding compound. By CFTR-binding compound, it is intended that the compounds to be identified by the present method will interact with a cell's CFTR, preferably a mutated CFTR such as  $\Delta$ F508, 35 such that the chloride ion efflux mediated by the CFTR will be increased, and perhaps restored. Such interaction between a CFTR-binding compound and the CFTR

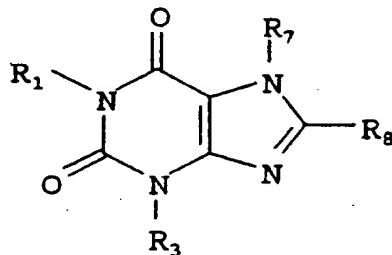
protein may be provided by any suitable combination of ionic, hydrophobic, and van der Waals bonds or forces, as a function of the sequence of the amino acids of the CFTR and the physico-chemical characteristics of the CFTR-  
5 binding compound. For the identification of such CFTR-binding compounds, preferably those that are candidates for testing as CF-directed therapeutic agents, it is useful to test for positive effect of affinity to polypeptide I $\alpha$  and, preferably, also for neutral or  
10 negative effect of no affinity to polypeptide I $\beta$  and/or I $\alpha$ . The conditions used for such affinity assays for identifying CFTR-binding compounds can be optimized using conventional techniques well known in the art. The method may identify any suitable compound that selectively binds  
15 to I $\alpha$ , for example, but not to I $\beta$ , for example, wherein the compound is a hydrocarbon, including, but not limited to, a protein, a lipid, a nucleic acid, or any combination thereof.

Among the effects of a CFTR-binding compound of interest are the following: (1) commences or causes the aggregation of synthetic polysaccharide liposomes when added to a preparation of (i.e., composition comprising) such liposomes (described in Example 1); (2) commences or causes the aggregation of chromaffin granules when added to a preparation of (i.e., composition comprising) such chromaffin granules (described in Example 1); and (3) commences or causes the aggregation of the aforementioned liposomes at an increased rate when an activator of chloride ion efflux of a  $\Delta$ F508 cell is added to such liposomes (see Figures 5-7). Activators of chloride ion efflux useful in the context of the present invention include CPX, caffeine, N,N-diallylcyclohexylxanthine, and other suitable compounds. No chloride ion efflux activator that has been tested in conjunction with I $\alpha$  has failed to exhibit the phenomenon of increased rate of liposome aggregation relative to the effect of I $\alpha$  alone.

The present invention further provides a method of treating cells having a reduced apical  $\text{Cl}^-$  conductance, such as cystic fibrosis cells, and provides novel compounds and pharmaceutical compositions thereof useful in the context of the present inventive method.

5 Specifically, the treatment method involves contacting cells having a reduced apical  $\text{Cl}^-$  conductance with a therapeutically effective quantity of a compound that has little or no affinity for adenosine cell receptors, particularly either the  $\text{A}_1$ -adenosine cell receptor or the  $\text{A}_2$ -adenosine cell receptor, yet stimulates  $\text{Cl}^-$  efflux. In 10 particular, the method involves contacting such cells with a compound having the formula

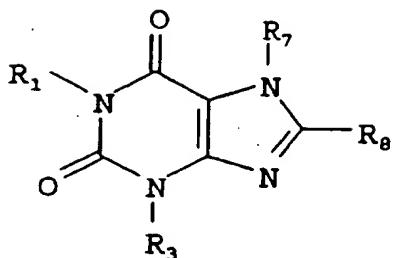
15



wherein  $\text{R}_1$  and  $\text{R}_3$  are the same and are  $\text{C}_1\text{-C}_6$  alkyl or  $\text{C}_1\text{-C}_6$  alkenyl,  $\text{R}_7$  is  $\text{C}_1\text{-C}_6$  alkyl or hydrogen, and  $\text{R}_8$  is  $\text{C}_4\text{-C}_8$  cycloalkyl, or a therapeutically effective derivative 20 thereof. Such compounds may be characterized with respect to their respective affinity for an adenosine receptor, such as the  $\text{A}_1$ - or  $\text{A}_2$ -adenosine receptor. In particular, the affinities of such compounds can be 25 expressed with respect to a  $K_i$  value, such as having a  $K_i$  of greater than or equal to 0.01. Preferred compounds have a  $K_i$  of at least 0.05. Such preferred compounds include those where  $\text{R}_1$  and  $\text{R}_3$  are methyl, propyl or allyl,  $\text{R}_7$  is methyl or hydrogen, and  $\text{R}_8$  is cyclopentyl or 30 cyclohexyl, and in particular are one of the group consisting of 1,3-dipropyl-7-methyl-8-cyclohexylxanthine, 1,3-dipropyl-7-methyl-8-cyclopentylxanthine, 1,3-diallyl-8-cyclohexylxanthine, and 8-cyclohexyl caffeine.

The present inventive method has particular utility in the treatment of cystic fibrosis cells. The method is especially preferred in the treatment of cystic fibrosis cells that have a deletion involving phenylalanine at 5 amino acid position 508 of the cystic fibrosis transmembrane regulator, in particular those cystic fibrosis cells found within a mammal, such as a human patient.

The compound used in the present inventive method is 10 preferably one that does not have phosphodiesterase activity. It is also preferred that the therapeutically effective quantity of the compound is nontoxic. Most preferably, the compound, itself, is nontoxic or, if toxic, provides a benefit that outweighs such toxicity. 15 It is further preferred that the compound of the present invention has a low level of affinity for any adenosine receptor (such as having a  $K_i$  of greater than or equal to about 0.01 mM) as recited above and exemplified below with respect to  $A_1$ - or  $A_2$ -adenosine receptors. With 20 respect to  $A_3$ -adenosine receptors, it is not likely the compounds of the present invention have substantial affinity thereto because most xanthines cannot be accommodated in the  $A_3$ -adenosine receptor binding site. van Galen et al., Medicinal Res. Rev., 12, 423-471 25 (1992). Especially preferred compounds for use in the present inventive method are 1,3-dipropyl-7-methylcyclopentylxanthine (DP-CPX), 1,3-diallyl-8-cyclohexylxanthine (DCHX), and cyclohexyl caffeine (CHC); the most preferred compound is DCHX. 30 Such compounds and pharmaceutical compositions containing same, heretofore undisclosed, are themselves embodiments of the present invention. In particular, compounds of the present invention first disclosed herein have the formula



wherein (a) R<sub>1</sub> and R<sub>3</sub> are the same and are methyl or  
 5 allyl, R<sub>7</sub> is ethyl, cyclopropylmethyl or hydrogen, and R<sub>8</sub> is cyclohexyl, provided that R<sub>1</sub> is allyl when R<sub>7</sub> is hydrogen and R<sub>1</sub> is methyl when R<sub>7</sub> is ethyl or cyclopropylmethyl, or (b) R<sub>1</sub> and R<sub>3</sub> are both methyl, and R<sub>7</sub> is hydrogen or methyl, and R<sub>8</sub> is cyclohexylmethyl or  
 10 cycloheptyl. A preferred compound according to the aforementioned structure is compound 17, i.e., 1,3-diallyl-8-cyclohexylxanthine (DCHX).

Alternatively, or additionally, a pharmaceutically acceptable derivative of DP-CPX, CHC, or DCHX, for  
 15 example, or combinations thereof as yet further examples, may be used in the present inventive method, which provide yet another embodiment of the present invention. It is desirable that such a derivative have equivalent therapeutic effectiveness in the context of the present  
 20 inventive method of treatment.

The compound useful in the present inventive method may be administered by any suitable means. One skilled in the art will appreciate that many suitable methods of administering the compound to an animal in the context of  
 25 the present invention, in particular a human, are available, and, although more than one route may be used to administer a particular compound, a particular route of administration may provide a more immediate and more effective reaction than another route.

30 The compound is preferably administered directly to the lung of a patient. Preferably, the compound is administered as a pharmaceutically acceptable aqueous solution. It is even more preferable that the compound

be administered as an aqueous pharmaceutical solution containing from about 0.001 to about 0.01% w/w of the compound. A pharmaceutically acceptable aerosol is another preferred means of administration. The aerosol 5 preferably contains from about 0.001 to about 0.01% w/w of the compound.

The compound also may be administered orally. In such a case, the compound will be generally administered in an amount of from about 0.1 to 1 mg/kg body weight per 10 day. A preferred amount of the inventive compound for oral administration is about 0.1 mg/kg body weight per day. Other routes of administration, such as intravenous and intraperitoneal administration, are also possible.

The compound should be administered such that a 15 therapeutically effective concentration of the compound is in contact with the affected cells of the body. The dose administered to an animal, particularly a human, in the context of the present invention should be sufficient to effect a therapeutic response in the animal over a 20 reasonable period of time. The dose will be determined by the strength of the particular compound employed and the condition of the animal, as well as the body weight of the animal to be treated. The size of the dose also 25 will be determined by the existence, nature, and extent of any adverse side effects that might accompany the administration of a particular compound and the particular route of administration employed with a particular patient. In general, the compounds of the present invention are therapeutically effective at low 30 doses. The effective dosage range is from about 30 nM to about 100 nM in the blood. Accordingly, the compounds will be generally administered in relatively low doses.

The compound may be administered in a pharmaceutically acceptable carrier. The present 35 invention encompasses pharmaceutical compositions comprising the present inventive compounds and pharmaceutically acceptable carriers. Pharmaceutically

acceptable carriers are well-known to those who are skilled in the art. The choice of carrier will be determined in part by the particular compound, as well as by the particular method used to administer the 5 composition. Accordingly, there is a wide variety of suitable formulations of the pharmaceutical composition of the present invention.

Formulations suitable for oral administration include (a) liquid solutions, such as an effective amount 10 of the compound dissolved in diluents, such as water or saline, (b) capsules, sachets or tablets, each containing a predetermined amount of the active ingredient, as solids or granules, (c) suspensions in an appropriate liquid, and (d) suitable emulsions. Tablet forms may 15 include one or more of lactose, mannitol, corn starch, potato starch, microcrystalline cellulose, acacia, gelatin, colloidal silicon dioxide, croscarmellose sodium, talc, magnesium stearate, stearic acid, and other excipients, colorants, diluents, buffering agents, 20 moistening agents, preservatives, flavoring agents, and pharmacologically compatible carriers. Lozenge forms can comprise the active ingredient in a flavor, usually sucrose and acacia or tragacanth, as well as pastilles comprising the active ingredient in an inert base, such 25 as gelatin and glycerin or sucrose and acacia emulsions, gels and the like containing, in addition to the active ingredient, such carriers as are known in the art.

Formulations suitable for administration by inhalation include aerosol formulations placed into 30 pressurized acceptable propellants, such as dichlorodifluoromethane, propane, nitrogen, and the like. The active agent may be aerosolized with suitable excipients.

Formulations suitable for intravenous and 35 intraperitoneal administration, for example, include aqueous and nonaqueous, isotonic sterile injection solutions, which can contain anti-oxidants, buffers,

bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and nonaqueous sterile suspensions that can include suspending agents, solubilizers, thickening 5 agents, stabilizers, and preservatives. The formulations can be presented in unit-dose or multi-dose sealed containers, such as ampules and vials, and can be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, 10 water, for injections, immediately prior to use. Extemporaneous injection solutions and suspensions can be prepared for sterile powders, granules, and tablets of the kind previously described.

The desirable extent of the induction of  $\text{Cl}^-$  efflux 15 from cells will depend on the particular condition or disease being treated, as well as the stability of the patient and possible side effects. In proper doses and with suitable administration of certain compounds, the present invention provides for a wide range of activation 20 of the rate of chloride ion efflux, e.g., from little activation to essentially full activation.

The present invention is expected to be effective in the treatment of all conditions, including diseases, that may be characterized by a reduced cellular apical  $\text{Cl}^-$  25 conductance. In particular, the present invention is expected to have utility in the treatment of chronic obstructive pulmonary diseases, especially cystic fibrosis.

The following examples serve to further illustrate 30 the present invention and are not intended to limit the scope of the invention.

#### EXAMPLE 1

This example illustrates the methods used to assess 35 the membrane-directed activity of certain CFTR polypeptides, and the results thereof.

5 Liposome aggregation assay: Phosphatidylserine ("PS", obtained from Avanti) was dissolved in chloroform, and small unilamellar liposomes were prepared by the extrusion process known in the art. A Lipex machine was  
10 used to extrude the PS in a buffer consisting of 10 mM Na-HEPES, pH 7.2 and 1 mM EDTA. The liposomes were diluted to a final optical density at 540 nm of 0.01 and incubated with different concentrations of the different polypeptides and/or chloride ion efflux-activating compounds.

15 Chromaffin granule aggregation assay: Adrenal glands were obtained from a local abattoir and transported to the laboratory on ice within 2 hours of 20 slaughter. Tissue was processed as described in Brocklehurst et al. in Polypeptide Hormones: A Practical Approach (Oxford Univ. Press, K. Siddle and J. Hutton, eds., 1990) at pp. 237-255. Chromaffin granules were purified on a step gradient of metrizamide using standard procedures. After resuspension, the granule aggregation assay was performed as described (Brocklehurst et al., supra), and the synexin activity was assessed from the initial rate of optical density increase at 540 nm. Briefly, the assay was carried out at room temperature in a 1 ml quartz cuvette containing the chromaffin granule 25 suspension in 0.3 mol/l sucrose, 40 mmol/l histidine buffer, pH 6.0, synexin and variable amounts of calcium chloride.

30 Preparation of CFTR polypeptides: Polypeptides were synthesized at the Yale University Department of Molecular Biology and Biophysics by standard solid phase methods. The polypeptides were purified by reverse phase HPLC, and the sequences were verified by direct chemical sequencing, amino acid analysis, and by mass spectroscopy, using standard methods well-known in the art. The specific polypeptides were I $\alpha$  [SEQ ID NO:1], I $\beta$  [SEQ ID NO:3], and I $\delta$  [SEQ ID NO:4].

Aggregation of chromaffin granules and liposomes by I $\alpha$  [SEQ ID NO:1]: As shown in Figure 2, polypeptide I $\alpha$  was found to drive aggregation of bovine chromaffin granules in the presence of 1 mM CaCl<sub>2</sub>. By contrast, the 5 contiguous polypeptide I $\beta$  [SEQ ID NO:3] was found to be essentially inactive in the chromaffin granule aggregation assay. Similarly, the overlapping polypeptide, I $\delta$  [SEQ ID NO:4], was also found to be inactive. These data thus appear to indicate that the 10 action of I $\alpha$  is specific to the I $\alpha$  sequence.

A titration of the I $\alpha$  activity, illustrated in Figure 3A, shows that the  $k_{1/2,app}$  was 22.2  $\mu$ g/ml or 6.3  $\mu$ M. A Hill plot of these data (see Figure 3B) indicated that the Hill coefficient ( $n_H$ ) was 2.6. Similar data 15 were observed using phosphatidylserine liposomes as an aggregation target, and a detailed titration over the pH range between pH 5.5 and 7.5 indicated that the pH for 50% activity was ca. 6.75 (see Figure 4A).

Aggregation of PS liposomes by low concentrations of I $\alpha$ : As shown in Figure 2, the aggregation reactions at high polypeptide concentrations were initiated promptly, and followed an apparent first order process that effectively plateaued within 5-10 minutes. The 20 cooperative character of the aggregation reaction driven by I $\alpha$  is quantitatively manifest by the Hill coefficient (see Figure 3A), which graphically shows that at lower polypeptide concentrations the initial rates of aggregation became small rather abruptly. However, at 25 much longer time periods it became evident that a significant aggregation reaction still occurred, albeit with complex kinetics. As shown in Figure 4B, the kinetics involved an initial lag phase of some 8-10 minutes, followed by a rising phase and a plateau. The conversion from an apparent first order process to the 30 more complex process occurred below 2  $\mu$ M of I $\alpha$  polypeptide, and the complex processes were clearly 35

evident in a dose dependent manner between 0.4 and 2  $\mu$ M. Below about 0.4  $\mu$ M, all activity virtually ceased.

Accordingly, I $\alpha$  drives the above-described membrane aggregation phenomena whereas the adjacent (I $\beta$ ) or 5 partially overlapping (I $\alpha$ ) polypeptide sequence, with respect to CFTR, does not drive such aggregation. The effective internal negative control clearly illustrates the existence of a special functional property located on the CFTR at the I $\alpha$  site, which property clearly 10 implicates membrane involvement.

#### EXAMPLE 2

This example illustrates the specific binding of certain chloride ion efflux activating compounds to I $\alpha$ , 15 and the inability of certain compounds that do not activate chloride ion efflux to bind to I $\alpha$ .

CPX was used as a representative compound known to activate chloride ion efflux from  $\Delta$ F508 mutant cells. Stock solutions of CPX were prepared in DMSO in the 20 millimolar concentration range. The I $\alpha$  polypeptide (100 nM final polypeptide concentration) was dissolved in 100  $\mu$ l of a buffer solution containing 50 mM TRIS (pH=7.0), 300 mM sucrose, and 10 mM glutathione. After 1 hour of incubation at 37°C, 1-100 nM  $^3$ H-CPX (ICN, specific 25 activity = 109 Ci/mole) was added and incubated for a further 15 minutes. For displacement studies, unlabeled CPX, or other xanthine antagonists and nucleotides, were added to the incubation mixture before the radioactive probe. The mixture was then filtered through a 30 nitrocellulose (NC) membrane (Schleicher & Schuell) by using a standard dot blot apparatus. Non-specific binding of  $^3$ H-CPX to NC filters was reduced by blocking filters with 0.3% polyethyleneimine (PEI). The dot blots were then washed five times with 200  $\mu$ l TRIS buffer-NaCl 35 solutions (50 mM TRIS pH=7.0, 150 mM NaCl), radioactive spots were cut out, and the total was radioactivity determined by liquid scintillation counting. Specific

$^3$ H-CPX binding to I $\alpha$  was determined by subtracting the non-specific binding, defined by preincubation with 10  $\mu$ M unlabelled CPX, from total binding.

5 The binding of  $^3$ H-CPX to I $\alpha$  was a saturable function of the CPX concentration. A fit of the data to a Scatchard plot showed the  $K_{D,app}$  to be 69 $\pm$ 27 nM (n=4). The  $B_{max}$ , estimated from the Scatchard plot intercept, indicated that the mole fraction of bound I $\alpha$  polypeptide was less than 1/2000. ~~Displacement of binding was~~

10 effected by CPX, caffeine, and DAX, all known chloride ion efflux activators, but was not effected by IBMX or 2-thio-CPX, which are known not to activate chloride ion efflux.

15 These data indicate that a chloride ion efflux activator can bind specifically to I $\alpha$ , thereby suggesting that the mechanism of CPX activation of cells bearing the CFTR ( $\Delta$ F508) mutation may be by interaction with the CFTR molecule itself. Moreover, because of the specificity of chloride ion efflux activators to bind and non-chloride ion efflux activators not to bind to I $\alpha$ , these data illustrate the utility of this method to identify further CFTR-binding proteins that may have a therapeutic effect on CF cells.

25

### EXAMPLE 3

This example illustrates the effect of CPX on PS liposome aggregation driven by I $\alpha$ .

30 The impact of CPX on PS liposome aggregation that was driven by I $\alpha$  was investigated. The study was done using concentrations of CPS of between 1 and 30 nM (activation noted in  $\Delta$ F508 containing cells), between 30 and 100 nM (maximum activation noted in the aforementioned cells), on up to 1000 nM (inhibition of activation noted in the aforementioned cells).

35 As shown in Figure 5, liposome aggregation driven by 0.5  $\mu$ g/ml (0.18  $\mu$ M) I $\alpha$  alone was very modest. However, with increases in CPX concentration, the aggregation

reaction was progressively activated after a lag of approximately 5 minutes. The maximum rate and extent of aggregation was observed at 100 nM CPX, and it thereafter fell when the CPX concentration was raised to 300 nM.

5 The inset to Figure 5 shows the extent of the reaction at a 20 minute time point.

Accordingly, these data show a parallel between the concentration-dependent behavior of CPX with (1)  $\Delta$ F508 containing cells and (2)  $I\alpha$  aggregation.

10

EXAMPLE 4

This example illustrates the effect of other xanthines on PS liposome aggregation driven by  $I\alpha$ . Included in the study were certain xanthine derivatives known to activate chloride ion efflux in  $\Delta$ F508 (e.g., caffeine and N,N-diallylcyclohexylxanthine (DAX)) or not to activate such chloride ion efflux (e.g., 2-thio-CPX and isobutylmethylxanthine (IBMX)).

20 Results from a PS liposome aggregation assay are shown in Figure 6. As is apparent from the graph, caffeine activates  $I\alpha$  aggregation with low potency, and does not appear to inactivate  $I\alpha$  aggregation at the highest concentrations. The inset to Figure 6 shows the extent of the reaction at the 20 minute time point, such 25 that the aggregation does not level off at all over the caffeine concentrations tested.

In the case of DAX, the potency is in the range of CPX, but no inactivation is seen at concentrations as high as 300 nM (see Figure 7). The inset to Figure 7 30 shows the extent of the reaction at a 20 minute time point. From this perspective, one can compare both CPX and DAX on the same type of plot (Figure 8), whereupon it can be seen that DAX is at least one-half a log less potent than CPX. However, the efficacies with respect to 35 the chloride ion efflux of DAX and CPX are virtually the same. Higher concentrations of DAX were not possible because of solubility limits.

Inclusion of 2-thio-CPX and IBMX, compounds known to be inactive as activators of chloride ion efflux from cells bearing the  $\Delta$ F508 mutation, in the liposome aggregation assay showed these compounds to be entirely 5 inactive at potentiating liposome aggregation driven by I $\alpha$  at concentrations up to 1 and 15  $\mu$ M, respectively.

Accordingly, the I $\alpha$ -driven PS liposome aggregation assay provides exactly parallel results with xanthine derivatives known to be active or inactive at chloride 10 ion efflux activation.

#### EXAMPLE 5

This example illustrates the effect of adenosine on I $\alpha$ -driven PS liposome aggregation. 15 Adenosine was included in a PS liposome aggregation assay. Adenosine had a modest but evident biphasic effect on the I $\alpha$ -driven liposome aggregation reaction. At the lower concentrations of 70 and 100 nM, adenosine modestly activated the aggregation reaction. However, at 20 higher concentrations, the activating effect was lost. At concentrations of 1  $\mu$ M adenosine and greater the aggregation reaction was actually suppressed below the already low control levels. These data thus indicate that the xanthines, CPX and DAX apparently interact with 25 a site on the I $\alpha$  polypeptide with affinity for adenosine.

#### EXAMPLE 6

This example illustrates the synthesis and chemical analysis of novel xanthine derivatives of the present 30 invention as well as of those previously disclosed.

Alkylation of the N-7 position in xanthine derivatives to provide compounds 13, 17, 21, and 23 was carried out as described in Jacobson et al., J. Med. Chem., 36, 2639-2644 (1993) and Daly et al., J. Med. Chem., 29, 1305-1308 (1986). Compounds 8-12, 14, 18, 19, 20, and 25 were synthesized as described in Jacobson et al. (Biochem. Pharmacol., 37, 3653-3661 (1988)); Shimada

et al. (J. Med. Chem., 35, 924-930 (1992)); and Shamim et al. (J. Med. Chem., 32, 1231-1237 (1989)); compounds 7, 14, 18, and 19 have been disclosed in Müll et al. (J. Med. Chem., 36, 3341-3349 (1993)) and Shamim et al. (J. Med. Chem., 35, 924-930 (1992)). Compounds 1-5 and 26, as well as 2-chloroadenosine, were purchased from Research Biochemicals International (Natick, MA). Compound 27 was disclosed in Thompson et al. (J. Med. Chem., 34, 2877-2882 (1991)).

TABLE I

Compound	R <sub>1</sub> ,R <sub>3</sub> =	R <sub>7</sub> =	R <sub>8</sub> =	K <sub>i</sub> , $\mu\text{M(A}_1\text{)}$	K <sub>i</sub> , $\mu\text{M(A}_2\text{)}$	Conc. max. (nM)	% control	thresh (nM)	% control
<u>8-unsubstituted</u>									
1 <sup>a</sup>	Me	H	H	8.5	25	100	150±30	1	130±20
2	Me	Me	H	29	48	10	170±20	1	120±30
3 <sup>b</sup>	Me,iBu <sup>c</sup>	H	H	7	16	>>10 <sup>4</sup>	—	—	—
<u>8-cyclopentyl</u>									
4 <sup>d</sup>	Me	H	cyclopentyl	0.011	1.4	>>10 <sup>4</sup>	—	—	—
5 <sup>c</sup>	Pr	H	cyclopentyl	0.00046	0.34	30	200±10	1	130±10
6	Pr	Me	cyclopentyl	2.3	11.4±1.4	1000	180±40	30	125±30
7	Pr,H <sup>f</sup>	H	cyclopentyl	0.014	0.58	30	160±40	1	130±20
8 <sup>e</sup>	Pr	H	cyclopentyl	0.00066	0.31	>>10 <sup>4</sup>	—	—	—
9	Pr	H	3-fluorocyclopentyl	0.042	—	>>300	—	—	—
10	Pr	H	3-iodocyclopentyl	0.058	—	>>300	—	—	—
11	Pr	H	cyclopenten-3-yl	0.045	0.640±0.061	>>300	—	—	—
12	Pr	H	noradamantyl	0.0013	0.38	10	170±20	1	120±5
<u>8-cyclohexyl</u>									
13	Me	H	cyclohexyl	0.030±0.015	1.04±0.20	3	160±20	0.1	130±20
14	Me	Me	cyclohexyl	28	17.1	3	150±30	3	150±30
15	Me	Et	cyclohexyl	6.66±0.99	3.23±0.36	>>10 <sup>4</sup>	—	—	—

TABLE 1

Compound	R <sub>1</sub> ,R <sub>3</sub> =	R <sub>7</sub> =	R <sub>8</sub> =	K <sub>i</sub> , $\mu\text{M}(A_1)$	K <sub>i</sub> , $\mu\text{M}(A_2)$	Conc. max. (nM)	% control	thresh (nM)	% control
16	Me	cyclopropylmethyl	cyclohexyl	8.23 $\pm$ 1.62	4.53 $\pm$ 1.37	>>10 <sup>4</sup>	—	—	—
17	allyl	H	cyclohexyl	0.0656 $\pm$ 0.0189	4.94 $\pm$ 0.68	10 <sup>3</sup>	260 $\pm$ 70	30	170 $\pm$ 40
18	Pr	H	cyclohexyl	0.0015	0.423 $\pm$ 0.055	>>10 <sup>4</sup>	—	—	—
19	Pr	Me	cyclohexyl	2.7	9.24 $\pm$ 0.37	5	170 $\pm$ 50	5	170 $\pm$ 50
20	Pr	H	cyclohexen-3-yl	0.010	0.905 $\pm$ 0.128	10 <sup>4</sup>	150 $\pm$ 30	10 <sup>4</sup>	150 $\pm$ 30
21	Me	H	cyclohexylmethyl	0.610 $\pm$ 0.076	5.47 $\pm$ 0.86	>>10 <sup>4</sup>	—	—	—
22	Me	Me	cyclohexylmethyl	3.71 $\pm$ 0.36	5.46 $\pm$ 0.47	>>10 <sup>4</sup>	—	—	—
<u>8-cycloheptyl</u>									
23	Me	H	cycloheptyl	0.0659 $\pm$ 0.0145	1.05 $\pm$ 0.19	>>10 <sup>4</sup>	—	—	—
24	Me	Me	cycloheptyl	19.5 $\pm$ 2.5	3.27 $\pm$ 0.98	>>10 <sup>4</sup>	—	—	—
<u>8-stryryl or 8-aryl</u>									
25 <sup>j</sup>	Me	Me	3-chlorostyryl	28	0.054	30	160 $\pm$ 30	30	160 $\pm$ 30
26 <sup>j</sup>	Pr	H	p- $\phi$ -OCH <sub>2</sub> CONH(CH <sub>2</sub> ) <sub>2</sub> NH <sub>2</sub>	0.0012	0.08	>>10 <sup>4</sup>	—	—	—
<u>non-xanthine antagonist</u>									
27	9-ethyl-6-cyclopentyladenine			0.44	17	>>10 <sup>4</sup>	—	—	—

<sup>a</sup>, theophylline; <sup>b</sup>, IBMX; <sup>c</sup>, 1-methyl-3-isobutyl; <sup>d</sup>, CPT; <sup>e</sup>, CPX; <sup>f</sup>, 1-propyl-3-H-analogue (Müller et al., *J. Med. Chem.*, **36**, 3341-3349 (1992)); <sup>g</sup>, 2-thio instead of oxo; <sup>h</sup>, KW-3902; <sup>i</sup>, CSC; <sup>j</sup>, XAC

An alkyl or cycloalkyl carboxylic acid was reacted with 5,6-diamino-1,3-dimethyluracil to obtain the corresponding amide, including 5-(cycloheptanoylamino)-6-amino-1,3-methyluracil and 5-(cyclohexylmethylcarbonyl)-6-amino-1,3-methyluracil, using the following protocol:

The acid (1 equiv) was dissolved in a minimum volume of N,N-dimethylformamide (DMF) containing 1,3-dialkyl-5,6-diaminouracil (1.5 equiv). 1-(3-Dimethylamino-propyl)-3-ethyl carbodiimide:HCl (1 equiv) was added, followed by a catalytic amount (0.05 equiv) of 4-(N,N-dimethylamino)pyridine and 0.05 equiv of imidazole. The mixture was stirred at room temperature for 3 hours, and saturated sodium chloride solution was added (for 1,3-dipropyl derivatives, water was used here) to form a precipitate or amorphous insoluble fraction. The insoluble residue was filtered and dissolved in 4 N aqueous sodium hydroxide containing sufficient methanol to obtain a clear solution. The mixture was heated at 60°C for 2 hours or until the complete disappearance of starting material, as judged using TLC (silica plate, CHCl<sub>3</sub>; CH<sub>3</sub>OH; HOAc; 85:10:5 v/v). The mixture was cooled and acidified to pH 1 with 6 N aqueous hydrochloric acid solution. The precipitate was washed with water, dried, and further purified using a preparative silica plate (85-95% CHCl<sub>3</sub>; 5-15% methanol; 1-5% HOAc).

The new compounds were characterized (and resonances assigned) by 300 MHz proton nuclear magnetic resonance mass spectrometry using a Varian GEMINI-300 FT-NMR spectrometer. Unless noted, chemical shifts are expressed as ppm downfield from tetramethylsilane. New compounds were characterized by chemical ionization mass spectrometry (NH<sub>3</sub>) on a mass spectrometer or in the EI mode using a VG7070F mass spectrometer. Representative spectral data for compounds 15, 16, 22, and 24 are: <sup>1</sup>H NMR CDCl<sub>3</sub> δ 3.42 (s, 3H N<sub>3</sub>CH<sub>3</sub>); 3.63 (s, 3H N<sub>5</sub>CH<sub>3</sub>); 3.89 (s, 6H OCH<sub>3</sub>); 5.06 (s, 2H, OCH<sub>2</sub>); 6.8 (s, 2H); 6.78 (d,

1H,  $J = 16$  Hz); 7.3-7.5 (m, 5H); 7.7 (D, 1H,  $J = 16$  Hz); MS (Cl) m/e 463 (MH<sup>+</sup> base), 375, 357.

Carbon, hydrogen, and nitrogen analyses were carried out by Atlantic Microlabs (Norcross, GA), and  $\pm 0.4\%$  was acceptable. A summary of the characterization studies conducted pursuant to application of the aforementioned techniques is provided in Table 2, where the formulas and elemental analyses of compounds 15-17 and 21-24 are presented.

10

TABLE 2

Compound	Yield %	M.p.(C)	Formula	Calculated			Found			
				C	H	N	C	H	N	
15	15	81	>170	C <sub>15</sub> H <sub>22</sub> N <sub>4</sub> O <sub>2</sub> .1/4H <sub>2</sub> O	61.10	7.69	19.00	61.25	7.48	18.59
	16	91	>120	C <sub>17</sub> H <sub>24</sub> N <sub>4</sub> O <sub>2</sub> .1/4H <sub>2</sub> O	63.63	7.70	17.46	63.62	7.57	17.16
	17	---	126-128	C <sub>17</sub> H <sub>22</sub> N <sub>4</sub> O <sub>2</sub> .1/2H <sub>2</sub> O	63.38	7.08	17.28	63.14	7.17	17.32
	21	74	236-238	C <sub>14</sub> H <sub>20</sub> N <sub>4</sub> O <sub>2</sub>	60.85	7.30	20.27	60.77	7.32	20.22
	22	73	158-159	C <sub>15</sub> H <sub>22</sub> N <sub>4</sub> O <sub>2</sub> .0.15H <sub>2</sub> O	61.48	7.67	19.12	61.76	7.83	18.72
	23	84	223-225	C <sub>14</sub> H <sub>20</sub> N <sub>4</sub> O <sub>2</sub>	60.85	7.30	20.27	60.67	7.31	20.22
20	24	89	199-201	C <sub>15</sub> H <sub>22</sub> N <sub>4</sub> O <sub>2</sub>	62.05	7.64	19.30	61.97	7.68	19.24

All xanthine derivatives were judged to be homogeneous using thin layer chromatography prior to biological testing.

25

#### EXAMPLE 7

This example describes the measurement of chloride efflux from CFPAC cells in response to contact between such cells and the xanthine derivatives of the present invention, including protocols required for culturing such cells and implementing such experimentation thereon.

CFPAC cells are pancreatic adenocarcinoma cells that are homozygous for the most common CFTR mutation, i.e., deletion of Phe<sup>508</sup> (Schoumacher et al., Proc. Natl. Acad. Sci., 87, 4012-4016 (1990)). CFPAC cells and CFTR-transfected CFPAC cells (CFPAC-4.7 CFTR) were obtained

from R. Frizzell at the University of Alabama. The cells were split and seeded at low density on 24-well COSTAR plates in medium composed of Iscove's medium, supplemented with 10% (vol/vol) heat-inactivated fetal calf serum, 100 units/ml penicillin, 100 mg/ml streptomycin, 0.25 mg/ml fungizone, and 1% (wt/vol) glutamine, and adjusted to an osmolarity of 310 mOsm. All culture materials were obtained from Biofluids (Rockville, MD). After 5 hours, the medium was replaced, and attached cells were allowed to grow to confluence during a period of 48-72 hours at 37°C in an atmosphere of 5% CO<sub>2</sub>/95% air.

Before each experiment, cells were loaded with [<sup>36</sup>Cl<sup>-</sup>] as follows. Confluent cells were washed four times in the medium disclosed in Example 6. Then, after aspirating the last wash, 500  $\mu$ l of medium containing about  $1.4 \times 10^8$  counts per minute (cpm) of [<sup>36</sup>Cl]<sup>-</sup> (Amersham) were added to each well. The plates of cells were then incubated at 37°C overnight in 5% CO<sub>2</sub>/95% air, thus allowing [<sup>36</sup>Cl]<sup>-</sup> isotopic equilibrium to be obtained. To initiate efflux experiments, concentrations of CPX and/or other xanthine derivatives were added to the cells, which were then incubated for 15 minutes at 19°C. After incubating, the cells were washed four times in successive aliquots of an ice-cold wash medium composed of 150 mM sodium-gluconate, 1.5 mM potassium gluconate, and 10 mM Na-Hepes (pH 7.4). At the end of the wash step, 500  $\mu$ l of bicarbonate-free flux medium at 19°C was added, and sampling was initiated by collecting 50  $\mu$ l aliquots from each well at 0, 1, 2, 3, 5, 7, and 10 minutes. The flux medium consisted of 150 mM sodium gluconate, 1.5 mM potassium gluconate, 10 mM sodium Na-Hepes (pH 7.4), 100  $\mu$ M bumetanide to inhibit efflux due to the cotransporter, and different concentrations of CPX or other drugs, e.g., A<sub>1</sub>-adenosine antagonists and activators of cAMP synthesis, as required and described

in subsequent examples. The osmolarity was 310 mOsm. At the end of each flux experiment, 20  $\mu$ l of 50% trichloroacetic acid was added to a final concentration of 5% to obtain a measure of remaining radioactivity.

5 Samples were mixed with 1.5 ml of Cytoscint fluid and assayed for two minutes on a Beckman LS9000 scintillation counter with windows at maximum width. Osmotic strength was measured by freezing point depression on an osmotic osmometer. See Eidelman et al., supra, 1992.

10 Table 1 summarizes the data derived from at least four repeats of the chloride efflux experiments using the xanthine derivatives of the present invention. Additionally, each data point within a repeated experiment was computed from the average of efflux

15 measurements performed on four separate cells. The column labeled "Conc. max (nM)" presents the concentration of the xanthine derivative at its maximum impact upon chloride efflux, and the succeeding column to the right presents the percentage of chloride efflux at maximum impact relative to control. The column labeled "thresh (nM)" presents the minimum concentration of the xanthine derivative required to detect an effect upon chloride efflux, and the succeeding column to the right presents the percentage of chloride efflux at minimum

20 detection relative to control. As can be seen in Table 1, the range of maximal stimulation is from about 3 nM (for compound 14, for example) to over  $10^4$  nM (for compound 3, for example). Percentage increases over control at the maximum stimulation concentrations ranged

25 from about 10% (i.e., non-effective compounds such as compounds 2 and 12) to about 260% +/- 70% (for compound 17). The concentration of the xanthine derivative at threshold stimulation of the chloride efflux ranged from 0.1 nM (for compound 13, for example) to about  $10^4$  nM

30 (for compound 20, for example). Percentage increases over control at the threshold stimulation concentrations

ranged from 120% (for compound 12) to about 170% (for compound 17, for example).

From these data, and in view of the preference of using a lesser concentration of any given drug to effect a desired result so as to minimize the incidence of undesired side effects, it is evident that compounds 6, 7, 13, 14, 17, 19, and 25 are preferred compounds in view of their noted ability to stimulate chloride efflux at  $\mu\text{M}$  or lesser concentrations.

Figures 9-12 present flux study data that is presented graphically for compound 1, i.e., theophylline (Figure 9); compound 5, i.e., CPX (Figure 10); compound 22, i.e., 8-cyclohexylmethyl caffeine (Figure 11); and compound 17, i.e., 1,3-diallyl-8-cyclohexylxanthine (Figure 12). The vertical axis in each of the graphs of Figures 9-12 represents the rate of  $[^{36}\text{Cl}]^-$  efflux (FA) of the radiolabeled chloride as a function of the concentration of the compound tested (horizontal axis). These were calculated by linear regression of the log (FA) as a function of time, using standard techniques, and are presented as a percentage relative to the rate constants of control wells on the same plate. Data were handled with a Quattro-Pro 4.0 program. The error bars represent the S.E.M. values of four experiments (i.e.,  $n=4$ ).

As can be seen by simple observation of Figures 9-12, each of compounds 1, 5, and 22 has the characteristic of inducing 200% of the efflux rate as compared to control. Compound 17 also demonstrates in excess of 100% of the efflux capability of the control, but to a lesser extent as compared to the other three compounds. Of the four compounds whose results are presented herein graphically, compounds 1 and 22 have little affinity for either tested adenosine receptor, compound 17 has moderate affinity for  $A_1$  receptors ( $K_i \approx 0.07$ ), and compound 5 has extreme affinity for  $A_1$  receptors ( $K_i \approx$

0.0005). Despite the fact that compound 17 has significantly less affinity for A<sub>1</sub>-receptors than compound 5, for example, compound 17 has been shown to be significantly more efficacious in its absolute chloride 5 efflux capability, as noted in Table 1 (a maximum of 260% of control for compound 17 versus 200% of control for compound 5).

#### EXAMPLE 8

10 This example illustrates assays for the detection of adenosine receptor binding and the results of such assays performed in partial characterization of the novel xanthine derivatives of the present invention.

15 Rat cerebral cortical membranes and striatal membranes were prepared according to the procedure of Hide et al., Mol. Pharmacol., **41**, 352-359 (1992) and treated with adenosine deaminase (2 U/ml) for 30 minutes at 37°C prior to storage at -70°C. Solid samples of the adenosine derivatives were dissolved in dimethyl 20 sulfoxide (DMSO) and stored in the art at -20°C. The stock solutions were diluted with DMSO to a concentration of ≤ 0.1 mM prior to addition to the aqueous medium. The final concentration of DMSO in the assay medium was generally 2%.

25 Inhibition of binding of 1 nM [<sup>3</sup>H]N<sup>6</sup>-phenylisopropyladenosine (Dupont NEN, Boston, MA) to A<sub>1</sub> receptors in rat cerebral cortex membranes was measured as described in Schwabe et al., Naunyn-Schmiedeberg's Arch. Pharmacol., **313**, 179-187 (1980), and Jacobson et al., J. Med. Chem., **36**, 1333-1342 (1993a). Membranes (~100 µg protein per tube) were incubated for 1.5 hours at 37°C in a total volume of 0.5 ml of 50 mM tris hydrochloride at pH 7.4. Test drugs were dissolved in DMSO and added in 10 µl aliquots, resulting in a final 35 DMSO concentration of 2%. Bound and free radioligand were separated by addition of 3 ml of a buffer containing

50 mM tris hydrochloride, at pH 7.4 at 5°C, followed by vacuum filtration using a Brandel Cell Harvester (Brandel, Gaithersburg, MD) and a Whatman GF/B glass fiber filter with additional washes totaling 9 ml of 5 buffer. Non-specific binding was determined with 10  $\mu$ M 2-chloroadenosine.

Inhibition of binding of 5 nM [ $^3$ H]CGS 21680 (2-[4-[(2-carboxyethyl)-phenyl]ethylamino]-5'-N-ethylcarboxamidoadenosine) (Dupont NEN, Boston, MA) to A<sub>2a</sub> receptors 10 was carried out as reported in Jarvis et al., J. Pharmacol. Exp. Therap., 251, 888-893 (1989). Membranes (~80  $\mu$ g protein per tube) were incubated for one hour at 25°C in a total volume of 0.5 ml of a buffer solution 15 consisting of 50 mM tris hydrochloride and 10 mM MgCl<sub>2</sub> at pH 7.4. Test drugs were dissolved in DMSO and added in 10  $\mu$ l aliquots, resulting in a final DMSO concentration of 2%. Non-specific binding was defined using 20  $\mu$ M 2-chloroadenosine. Filtration was carried out using a Brandel Cell Harvester, as above, using the 20 tris-HCl/MgCl<sub>2</sub> buffer recited hereinabove as the washing buffer.

At least six different concentrations spanning three orders of magnitude, adjusted appropriately for the IC<sub>50</sub> 25 of each compound, were used. IC<sub>50</sub> values, computer-generated using a non-linear regression formula on the GraphPAD program (Institute for Scientific Information), were converted to apparent K<sub>i</sub> values using K<sub>D</sub> values (Jacobson et al., supra, 1993a; Ukena et al., FEBS Letters, 209, 122-128 (1986)) of 1.0 and 14 nM for 30 [ $^3$ H]PIA and [ $^3$ H]CGS 21680 binding, respectively, and the method of Cheng et al. (Biochem. Pharmacol., 22, 3099-3108 (1973)).

The results of the adenosine receptor binding 35 studies are presented in Table 1 for all of the xanthine derivatives under the columns "K<sub>i</sub>,  $\mu$ M (A<sub>1</sub>)" and "K<sub>i</sub>,  $\mu$ M (A<sub>2</sub>). Receptor antagonists having a high affinity for a

particular receptor are characterized by having very low  $K_i$  values, as determined using the assays described above for the  $A_1$  and  $A_2$  receptors. Accordingly, it can be seen that compound 5 has high affinity for the  $A_1$  receptor  
5 whereas compound 6 has low affinity for either of the receptors tested.

Figures 13-14 also depict data derived from the receptor studies described above, with particular reference to ascertaining the effects of changing ring 10 size of the 8-cycloalkyl substituent on binding at adenosine receptors. Values for rat cortical  $A_1$ -receptors (closed circles, ●) and rat striatal  $A_{2a}$ -receptors (open triangles, Δ) are incorporated into the graphs. The affinity of 8-cycloalkyltheophylline 15 derivatives (Figure 16) and the affinity of 8-cycloalkylcaffeine derivatives (Figure 17) at  $A_1$ - and  $A_{2a}$ -receptors are shown. Values are given as the average of two or three (+/- s.e.m.) determinations performed in triplicate. Data for 8-cyclobutyl derivatives are from 20 Jacobson et al., J. Med. Chem., 36, 2639-2644 (1993b).

Figures 13 and 14 demonstrate that among derivatives of theophylline (1,3-dimethylxanthine), the cyclopentyl analogue, compound 4, had the highest  $A_1$  affinity. Among derivatives of caffeine (1,3,7-trimethylxanthine), the 25 cycloheptyl analogue, compound 24, showed some  $A_{2a}$  affinity (6-fold), whereas smaller ring sizes (4-6) had little to no affinity (Jacobson et al., supra, 1993b). These structure activity relationships diverge greatly from those observed for the same compounds in stimulating 30  $Cl^-$  efflux.

The results provided above, when considered *in toto*, indicate that compounds 6, 14, 17, and 19 provide the characteristics of substantial efflux activity at low concentration ranges coupled to a lack of activity at the 35 adenosine receptors.

The results of these examples indicate that it is possible to (1) identify compounds that activate cellular chloride ion efflux using an in vitro liposome- and/or CFTR-binding assay; and (2) activate chloride efflux from 5 CF cells by using a compound that has little or no affinity for the A<sub>1</sub>- or the A<sub>2</sub>-adenosine receptor, such as compounds 6, 14, 17, or 19. The most efficacious compound tested was compound 17. The therapeutic advantages of a drug that is able to activate chloride 10 efflux from CF cells specifically without having an impact or substantial impact at adenosine receptors are appreciable, given that such drug action would be less encumbered by undesirable side effects to other tissues.

15 All of the references identified herein, including patents, patent applications, and publications, are hereby incorporated by reference in their entireties.

20 While the invention has been described with an emphasis upon preferred embodiments, it will be obvious to those of ordinary skill in the art that variations in the preferred method, compound, and composition can be used and that it is intended that the invention can be practiced otherwise than as specifically described herein. Accordingly, this invention includes all 25 modifications encompassed within the spirit and scope of the invention as defined by the following claims.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: THE UNITED STATES OF AMERICA, REPRESENTED BY THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES
- (ii) TITLE OF INVENTION: A METHOD OF IDENTIFYING CFTR-BINDING COMPOUNDS USEFUL FOR ACTIVATING CHLORIDE CONDUCTANCE IN ANIMAL CELLS
- (iii) NUMBER OF SEQUENCES: 6
- (iv) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (v) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER: WO
  - (B) FILING DATE: 06-NOV-1995
  - (C) CLASSIFICATION:
- (vi) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER: US 343714
  - (B) FILING DATE: 22-NOV-1994

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 32 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: polypeptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Pro	Ser	Glu	Gly	Lys	Ile	Lys	His	Ser	Gly	Arg	Ile	Ser	Phe	Cys	Ser
1					5						10			15	
Gln	Phe	Ser	Trp	Ile	Met	Pro	Gly	Thr	Ile	Lys	Glu	Asn	Glu	Glu	Phe
	20							25					30		

## (2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 96 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: mRNA (genomic)

44

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CCNUCNGARG GNAARAUHAA RCAYUCNGGN CGNAUHUCNU UYUGYUCNCA RUUYUCNUGG	60
AUHAUGCCNG GNACNAUHAA RGARAAYGAR GARUUY	96

## (2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: polypeptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Gly Val Ser Tyr Asp Glu Tyr Arg Tyr Arg Ser Val Ile Lys Ala Cys			
1	5	10	15
Gln Leu Glu Glu Asp Ile Ser Lys Phe Ala Glu Lys Asp Asn Ile			
20	25	30	

## (2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 32 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: polypeptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Gly Thr Ile Lys Glu Asn Glu Glu Phe Gly Val Ser Tyr Asp Glu Tyr			
1	5	10	15
Arg Tyr Arg Ser Val Ile Lys Ala Cys Gln Leu Glu Glu Asp Ile Ser			
20	25	30	

## (2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 93 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: mRNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GGNGUNUCNU AYGAYGARUA YCGNUAYCGN UCNGUNAUHA ARGCNUGYCA RUURGARGAR	60
GAYAUHUCNA ARUUYGCNGA RAARGAYAAY AUH	93

45

## (2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 96 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: mRNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGNACNAUHA ARGARAAYGA RGARUUYGGN GUNUCNUAYG AYGARUAYCG NUAYCGNUCN	60
GUNAUHAARG CNUGYCARUU RGARGARGAY AUHUCN	96

WHAT IS CLAIMED IS:

1. A polypeptide selected from the group comprising polypeptide I $\alpha$  [SEQ ID NO:1], polypeptide I $\beta$  [SEQ ID NO:3], and polypeptide I $\omega$  [SEQ ID NO:4].

2. The polypeptide of claim 1, wherein said polypeptide is linked covalently to an affinity chromatography matrix.

3. The polypeptide of claim 2, wherein said affinity chromatography matrix is sepharose, agarose, polyacrylamide, or cellulose.

4. A method for identifying a CFTR-binding compound comprising contacting a putative CFTR-binding compound with polypeptide I $\alpha$  [SEQ ID NO:1] under conditions sufficient to allow for binding of said putative CFTR-binding compound to said polypeptide I $\alpha$ , and determining whether such binding occurred, an indication that said putative CFTR-binding compound is a CFTR-binding compound.

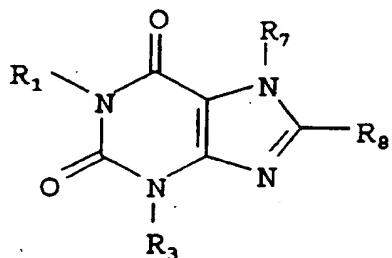
5. The method of claim 4, wherein said CFTR-binding compound has a neutral or negative affinity for polypeptide I $\beta$  [SEQ ID NO:3] or polypeptide I $\omega$  [SEQ ID NO:4].

6. The method of claim 4, wherein said CFTR-binding compound causes the aggregation of liposomes when added to a composition comprising said liposomes.

7. The method of claim 4, wherein said CFTR-binding compound causes the aggregation of chromaffin granules when added to a composition comprising said chromaffin granules.

8. The method of claim 7, wherein said composition consists essentially of said chromaffin granules in 1 mM CaCl<sub>2</sub>.

9. A method of treating cystic fibrosis in a mammal in need of such treatment, comprising administering a therapeutically effective amount of a compound having the formula



wherein  $R_1$  and  $R_3$  are the same and are  $C_1$ - $C_6$  alkyl or  $C_1$ - $C_6$  alkenyl,  $R_7$  is  $C_1$ - $C_6$  alkyl or hydrogen, and  $R_8$  is  $C_4$ - $C_8$  cycloalkyl, and wherein the  $K_i$  with respect to said compound and an adenosine receptor is at least 0.01.

10. The method of claim 9, wherein  $R_1$  and  $R_3$  are methyl, propyl, or allyl,  $R_7$  is methyl or hydrogen, and  $R_8$  is cyclopentyl or cyclohexyl.

11. The method of claim 9, wherein said mammal is a human.

12. The method of claim 11, wherein said compound is selected from the group consisting of 1,3-dipropyl-7-methyl-8-cyclopentylxanthine, 1,3-dipropyl-7-methyl-8-cyclohexylxanthine, 1,3-diallyl-8-cyclohexylxanthine, and 8-cyclohexyl caffeine.

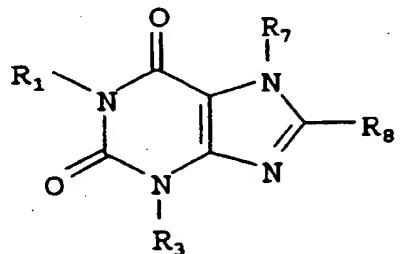
13. The method of claim 12, wherein said compound is 1,3-diallyl-8-cyclohexylxanthine.

14. The method of claim 9, wherein said compound is administered to the lung of said human.

15. The method of claim 14, wherein said compound is administered as an aqueous pharmaceutical composition containing from about 0.001 to about 0.01% w/w of said compound.

16. The method of claim 9, wherein said  $K_i$  is at least 0.05.

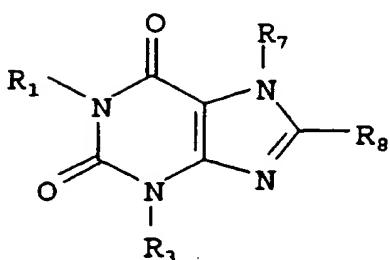
17. A compound having the formula



wherein (a)  $R_1$  and  $R_3$  are the same and are methyl or allyl,  $R_7$  is ethyl, cyclopropylmethyl or hydrogen, and  $R_8$  is cyclohexyl, provided that  $R_1$  is allyl when  $R_7$  is hydrogen and  $R_1$  is methyl when  $R_7$  is ethyl or cyclopropylmethyl, or (b)  $R_1$  and  $R_3$  are both methyl, and  $R_7$  is hydrogen or methyl, and  $R_8$  is cyclohexylmethyl or cycloheptyl.

18. The compound of claim 17, wherein said compound is 1,3-diallyl-8-cyclohexylxanthine.

19. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and a compound having the formula



wherein (a)  $R_1$  and  $R_3$  are the same and are methyl or allyl,  $R_7$  is ethyl, cyclopropylmethyl or hydrogen, and  $R_8$  is cyclohexyl, provided that  $R_1$  is allyl when  $R_7$  is hydrogen and  $R_1$  is methyl when  $R_7$  is ethyl or cyclopropylmethyl, or (b)  $R_1$  and  $R_3$  are both methyl, and  $R_7$  is hydrogen or methyl, and  $R_8$  is cyclohexylmethyl or cycloheptyl.

20. The pharmaceutical composition of claim 19, wherein said compound is 1,3-diallyl-8-cyclohexylxanthine.

21. A polynucleotide selected from the group consisting of polynucleotide  $I\alpha$  [SEQ ID NO:2], polynucleotide  $I\beta$  [SEQ ID NO:5], and polynucleotide  $I\circ$  [SEQ ID NO:6].

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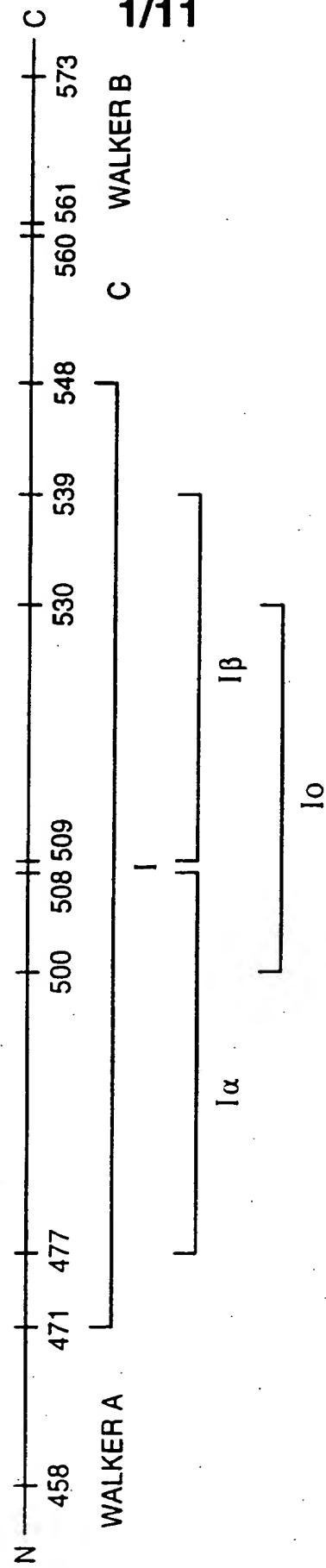


FIG. 1

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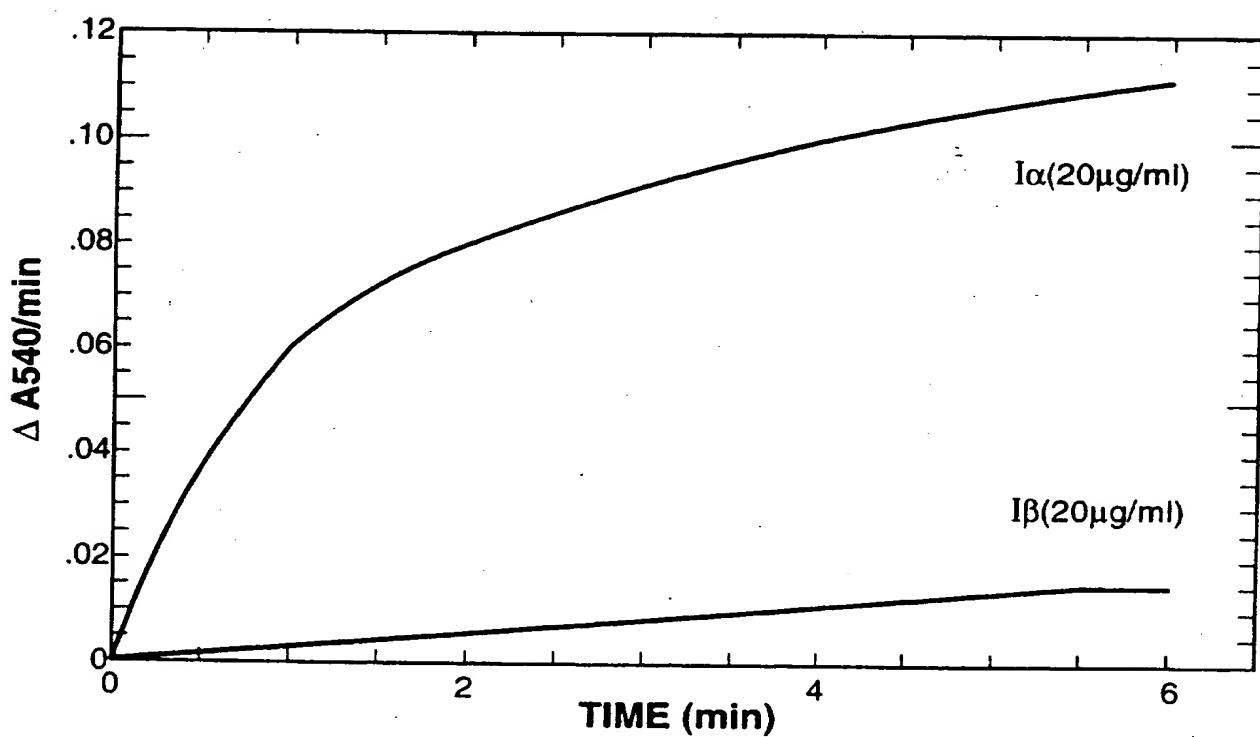


FIG. 2

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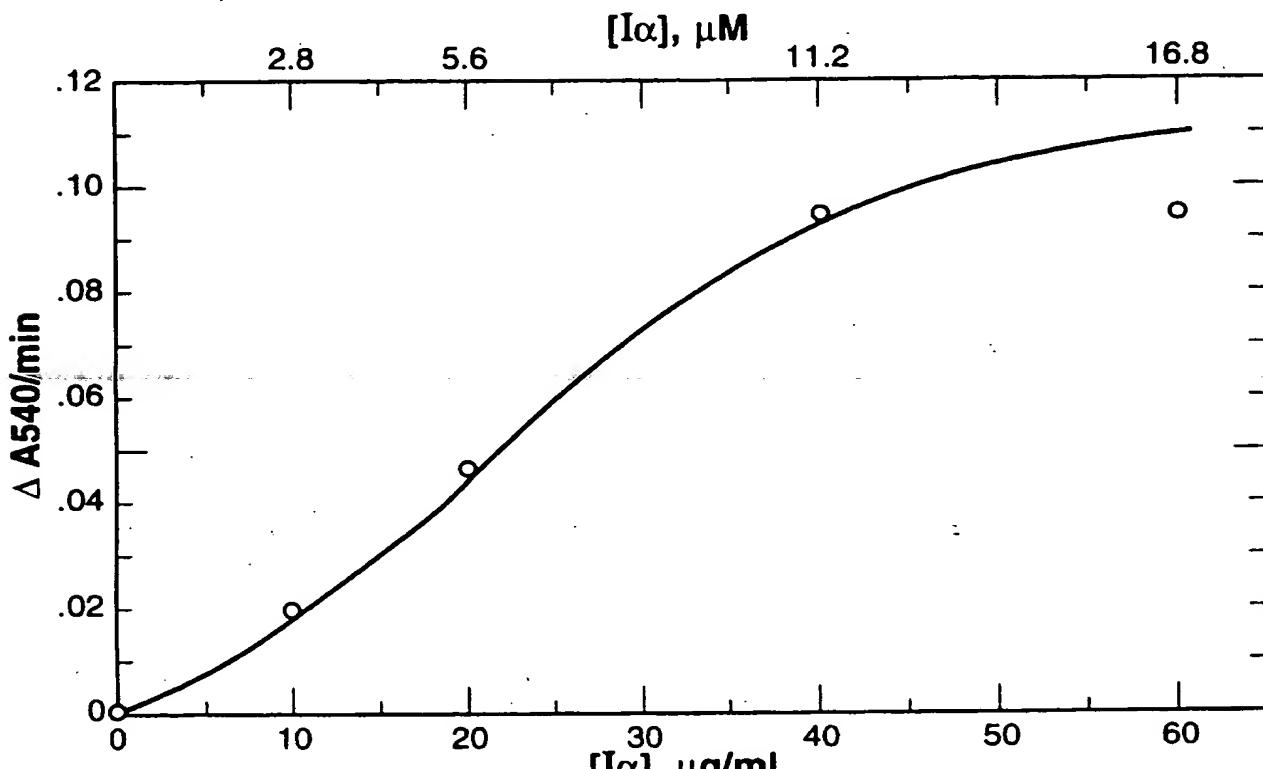


FIG. 3A

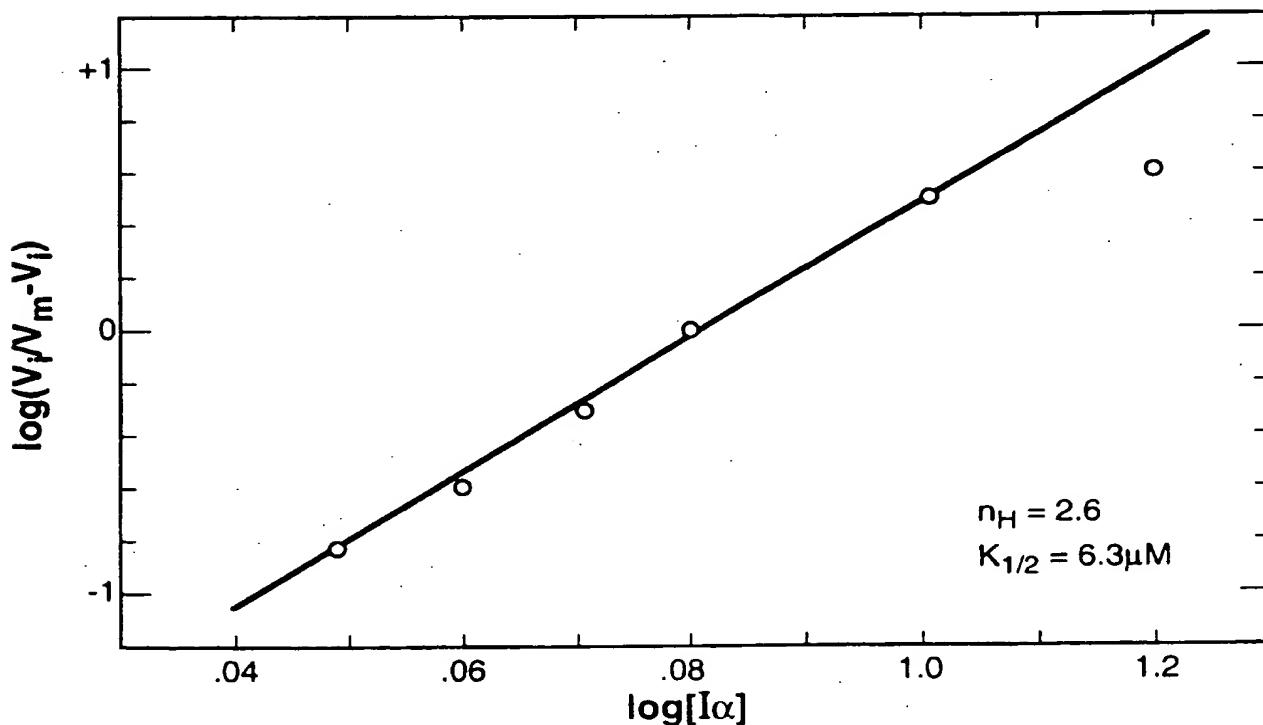


FIG. 3B

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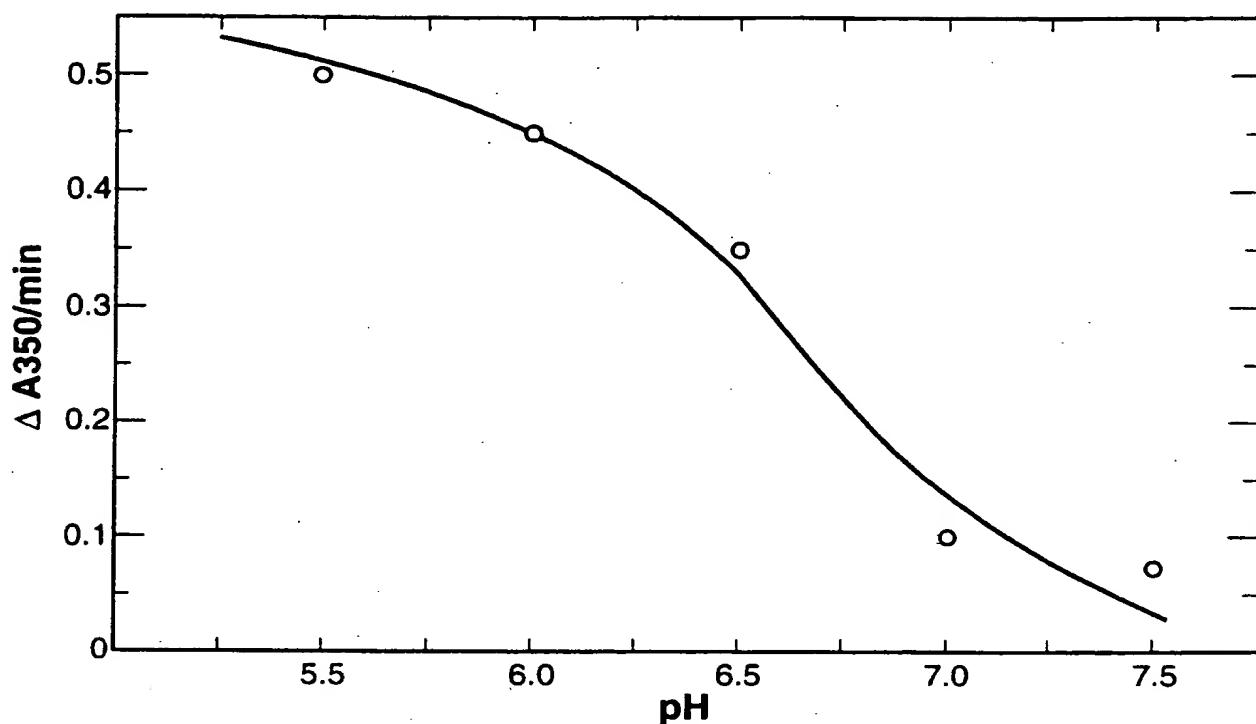


FIG. 4A

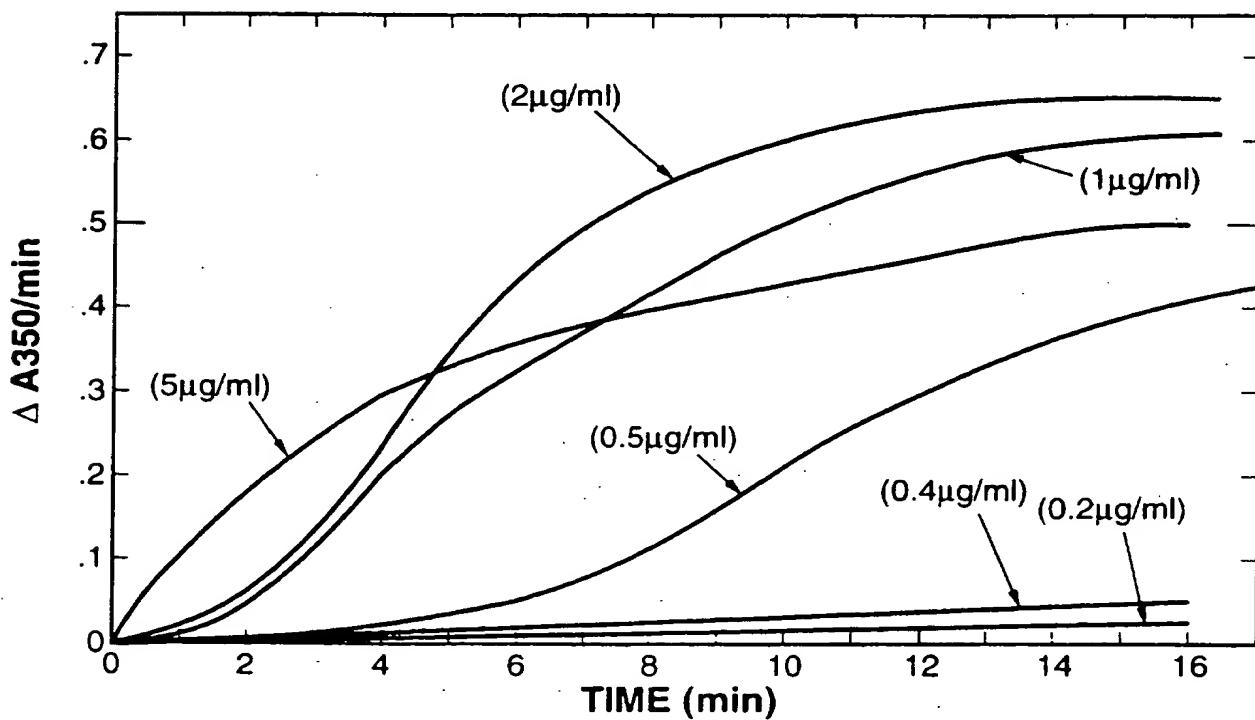


FIG. 4B

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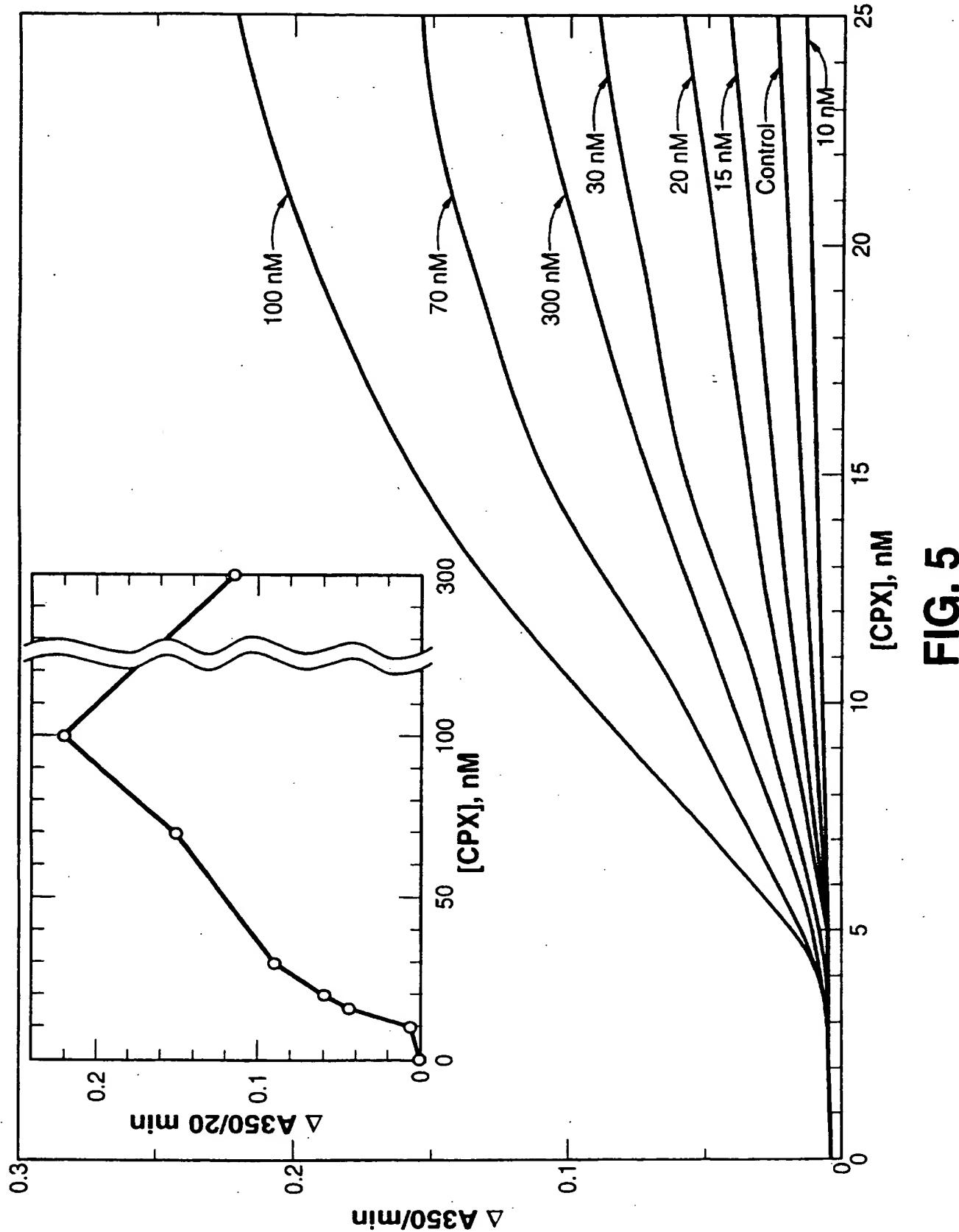


FIG. 5

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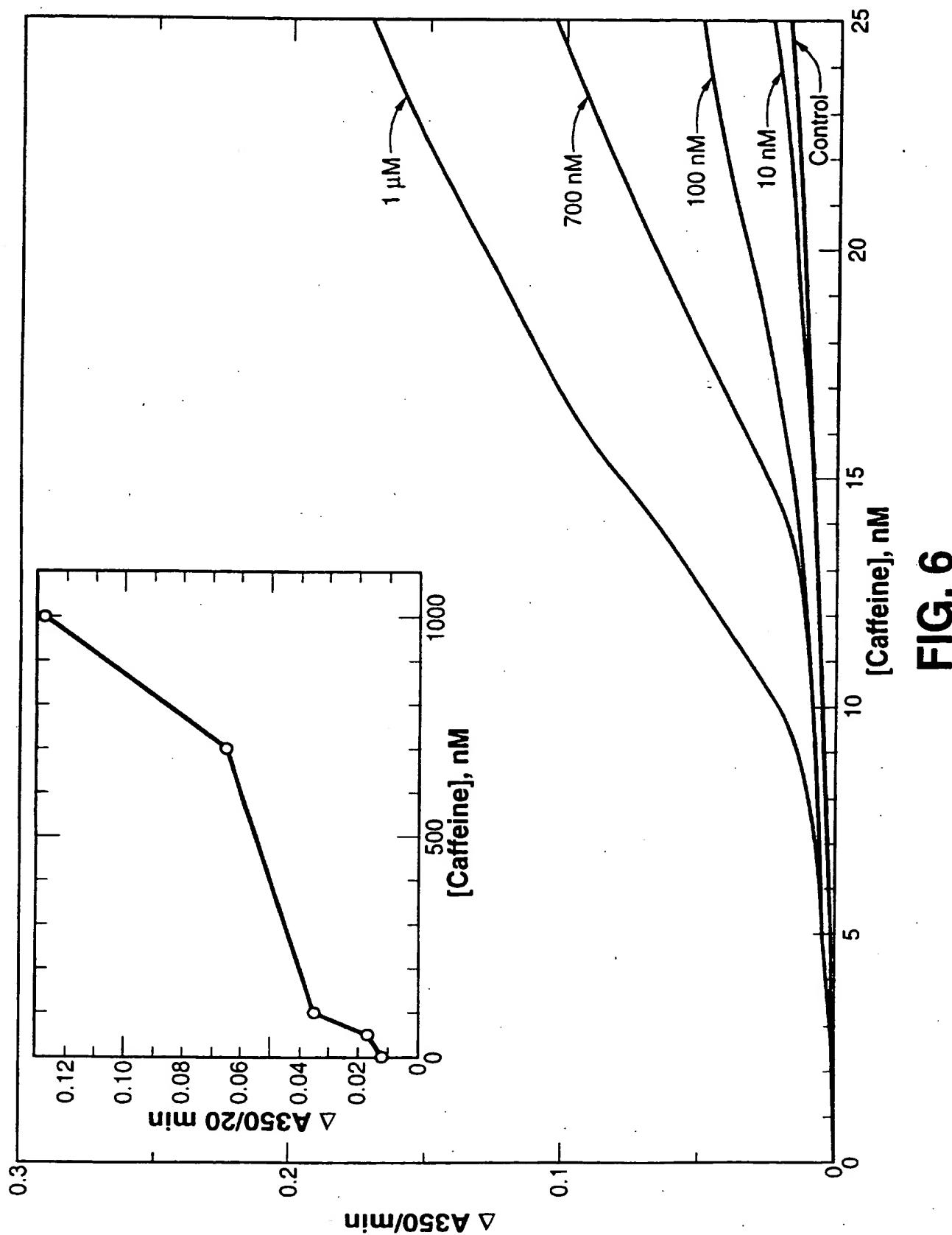


FIG. 6

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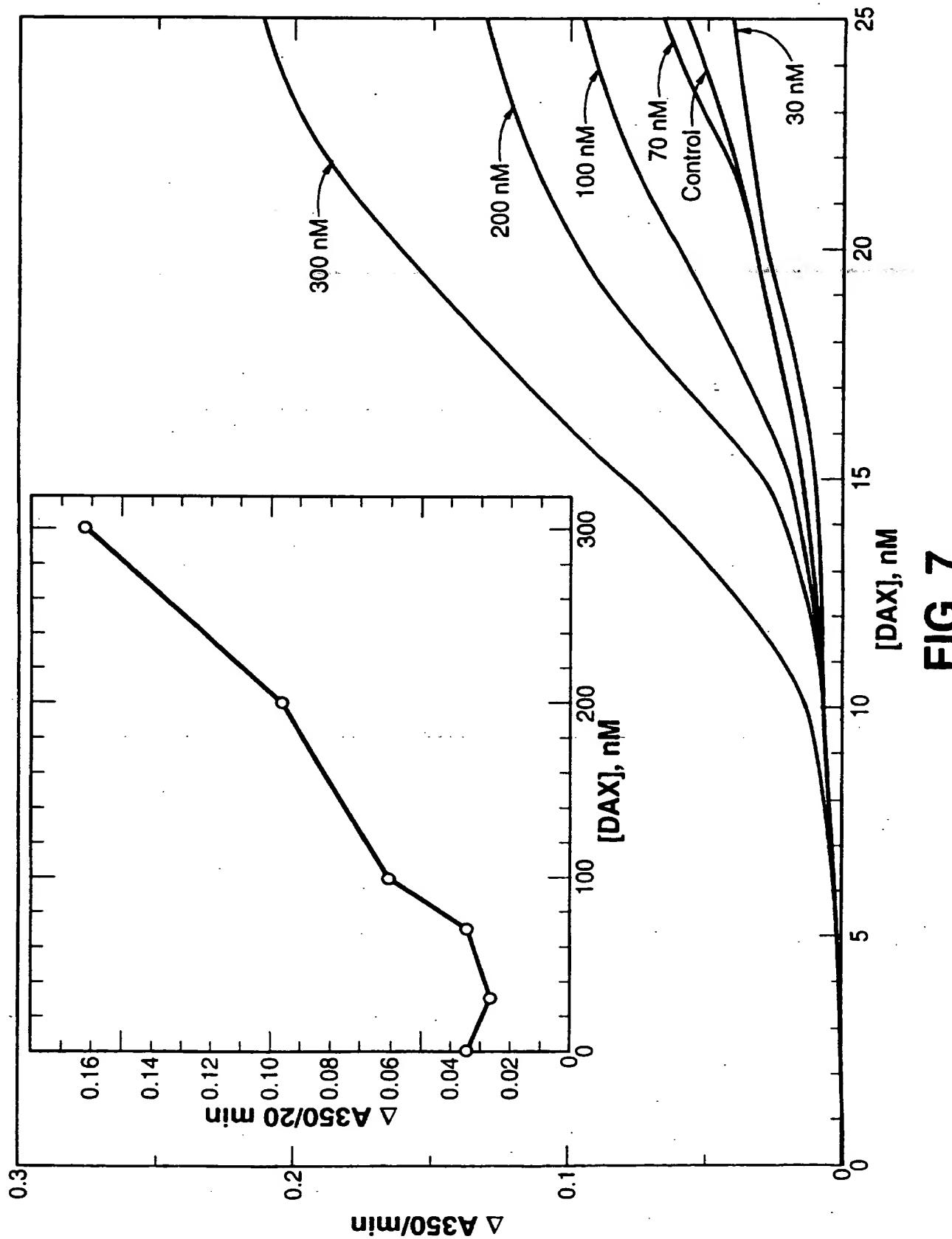


FIG. 7

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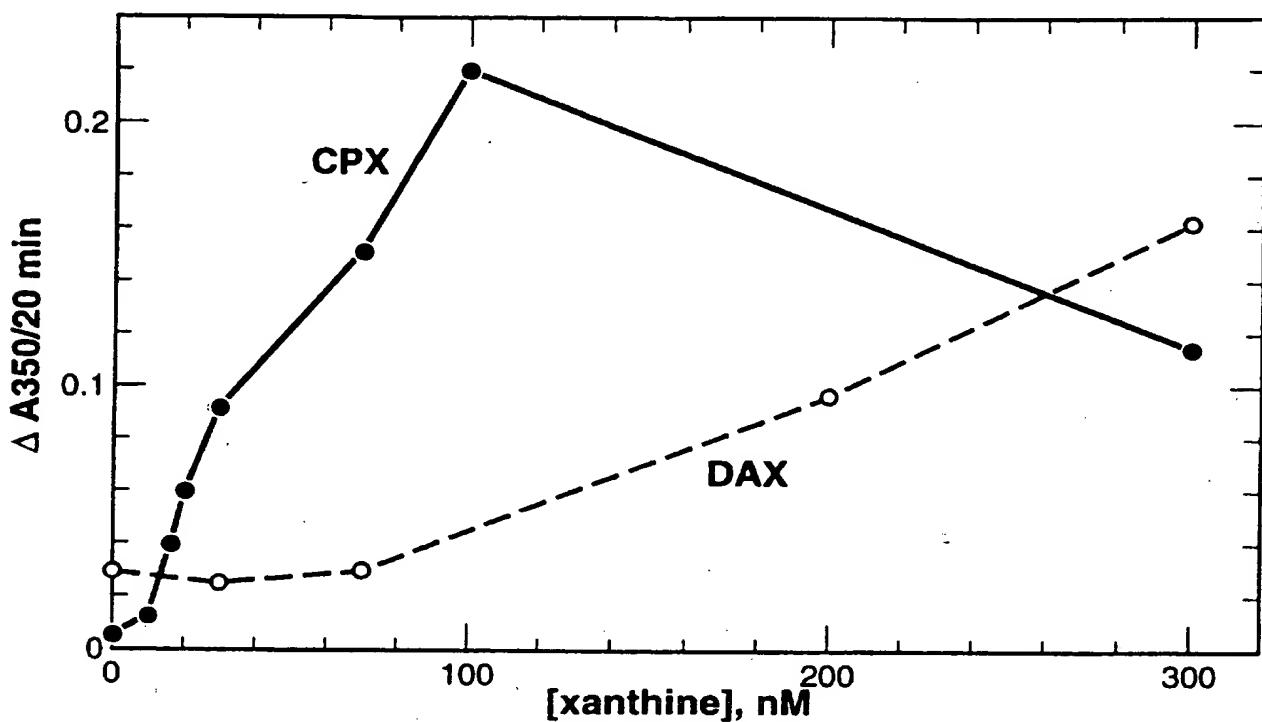


FIG. 8

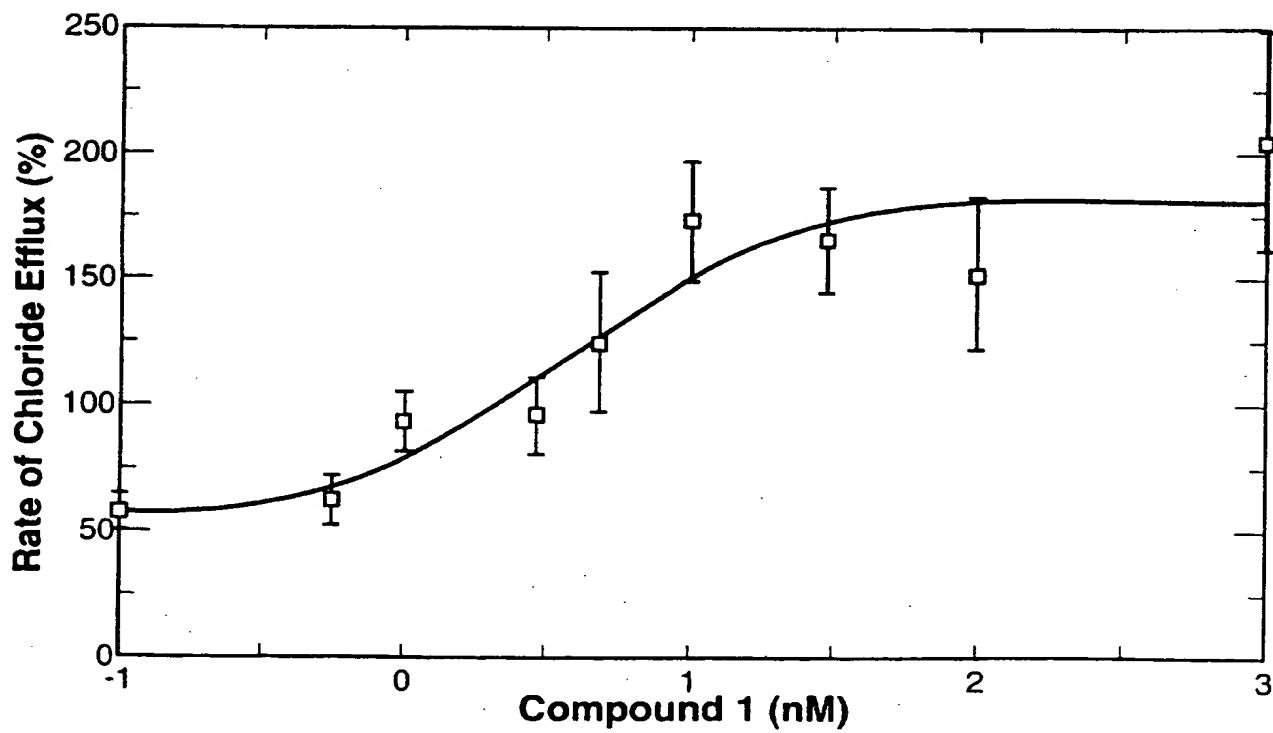


FIG. 9

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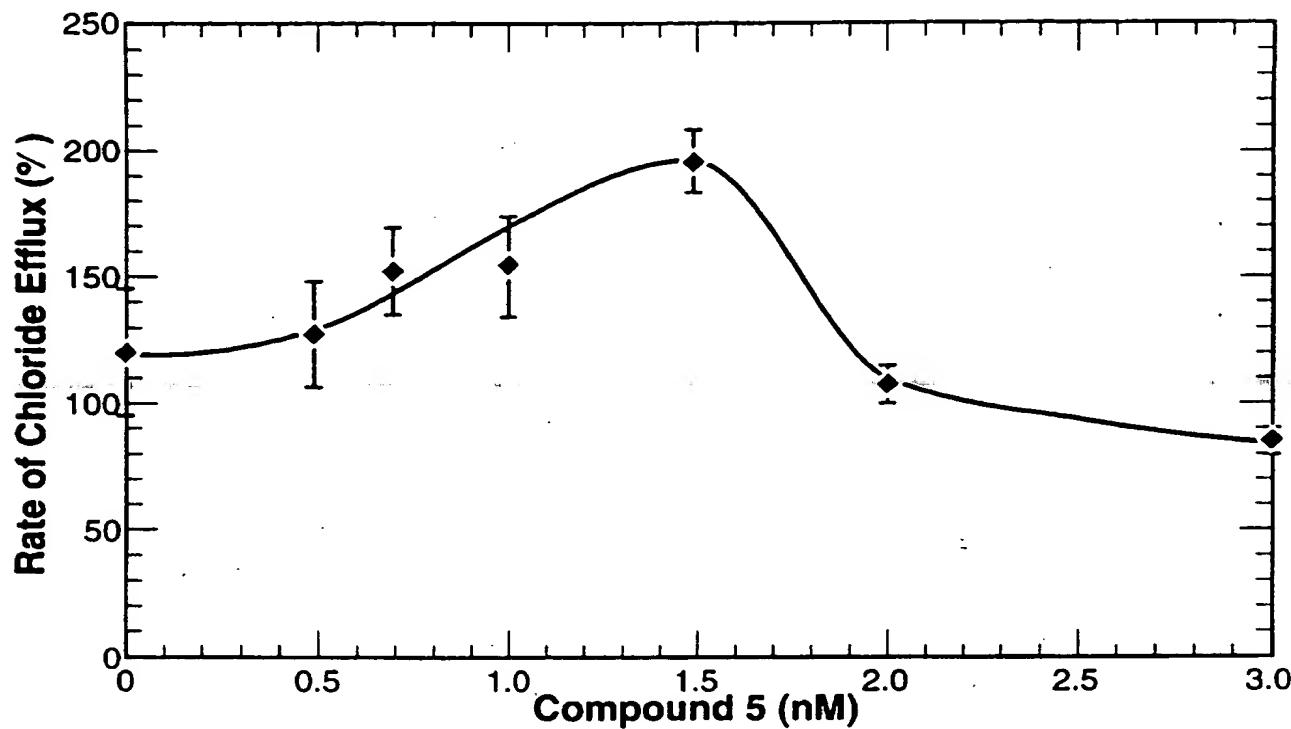


FIG. 10

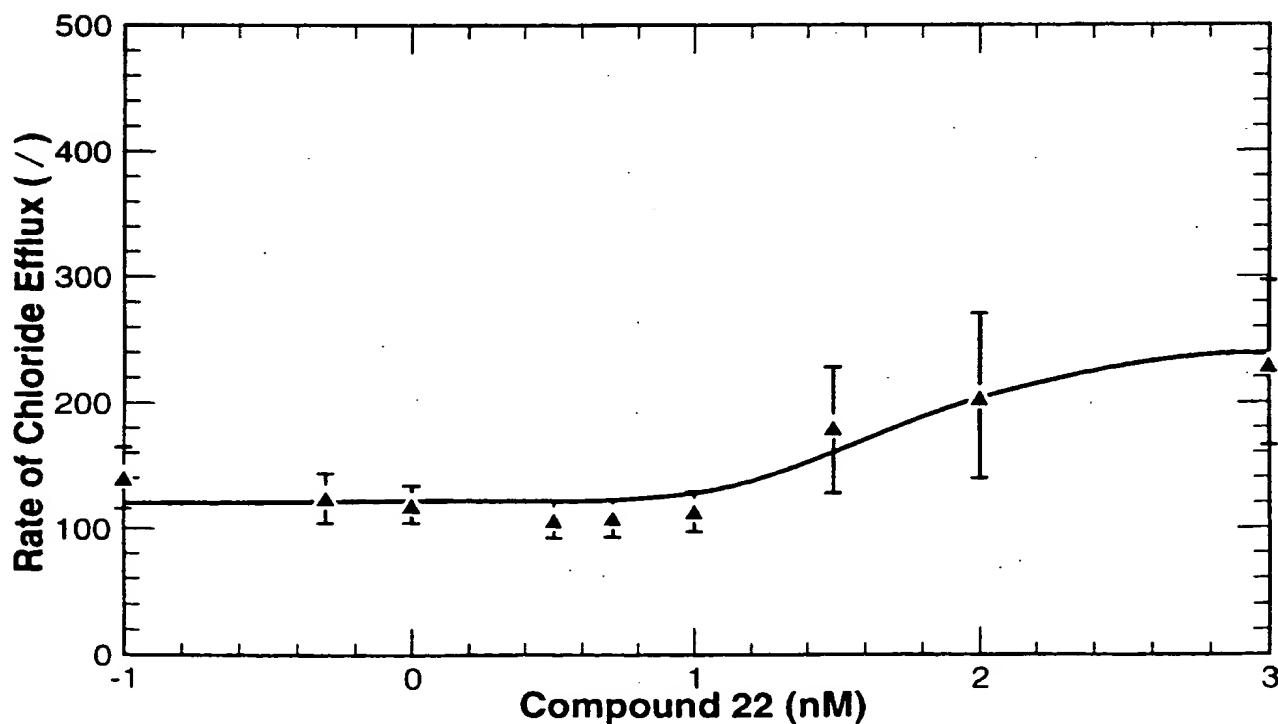


FIG. 11

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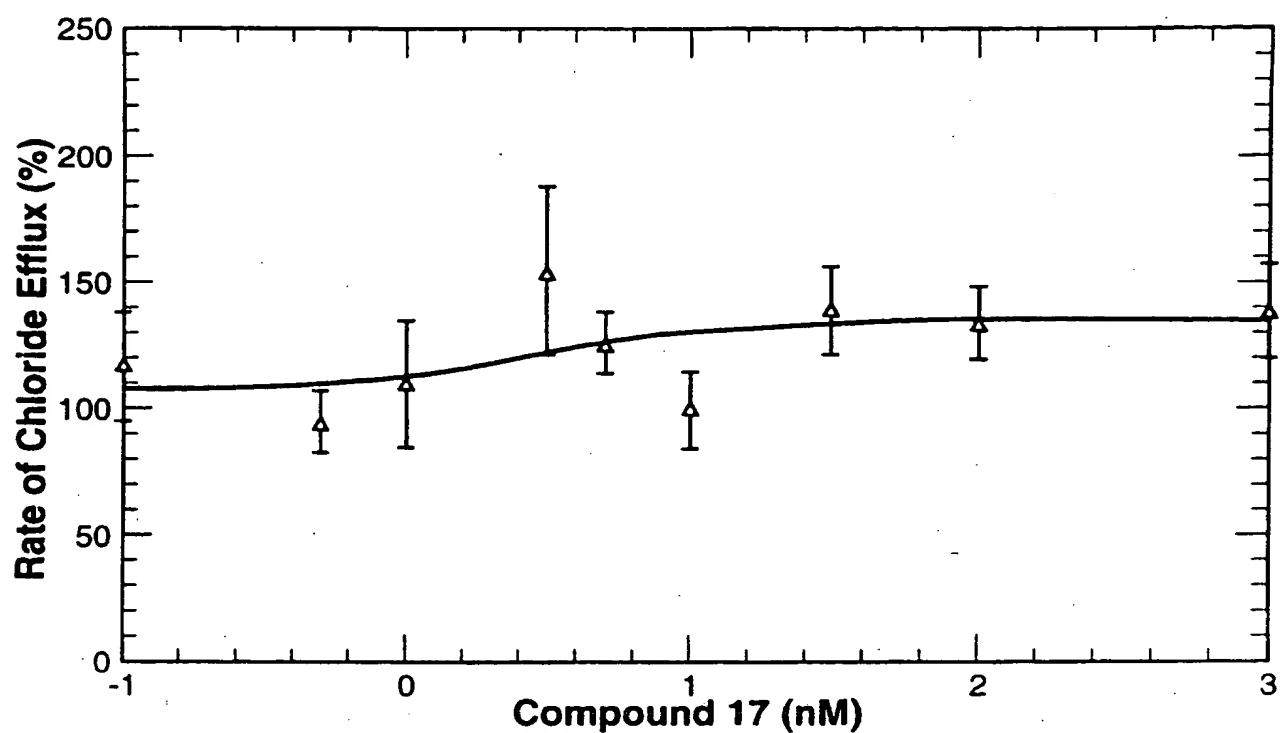


FIG. 12

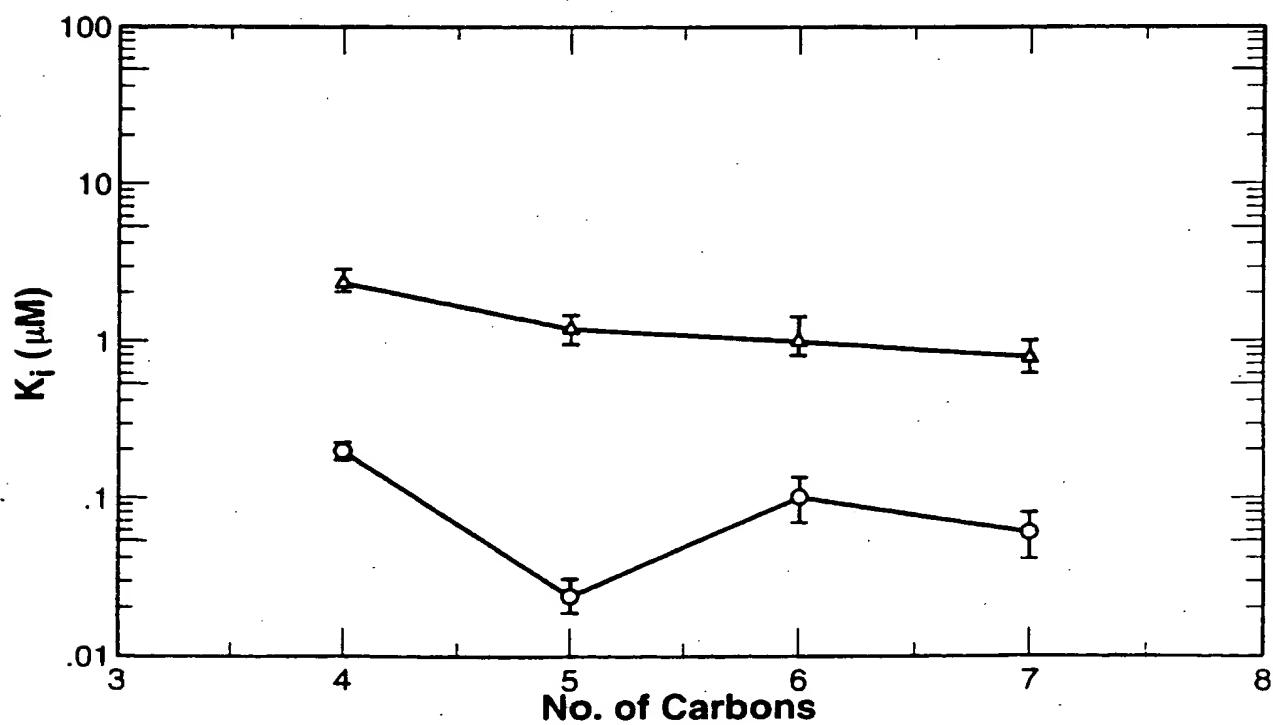


FIG. 13

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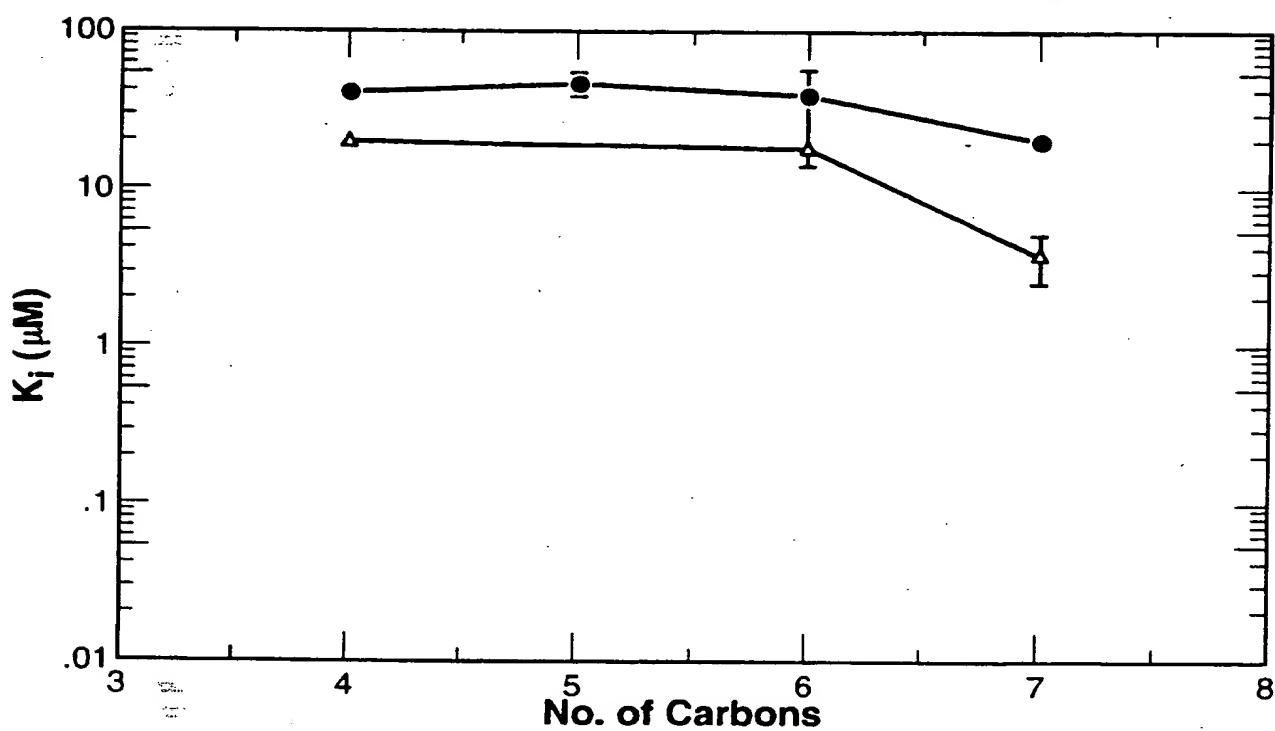


FIG. 14

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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification 6 :</b> <b>C07K 14/47, C07D 473/04, A61K 38/17, 31/52</b>		<b>A3</b>	<b>(11) International Publication Number:</b> <b>WO 96/16084</b> <b>(43) International Publication Date :</b> <b>30 May 1996 (30.05.96)</b>
<b>(21) International Application Number:</b> <b>PCT/US95/14345</b> <b>(22) International Filing Date:</b> <b>6 November 1995 (06.11.95)</b>  <b>(30) Priority Data:</b> <b>08/343,714</b> <b>22 November 1994 (22.11.94)</b> <b>US</b>		<b>(81) Designated States:</b> AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, LS, MW, SD, SZ, UG).	
<b>(71) Applicant:</b> THE UNITED STATES OF AMERICA, represented by THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICESNational Institutes of Health [US/US]; Office of Technology Transfer, Box OTT, Bethesda, MD 20892-9902 (US).  <b>(72) Inventors:</b> POLLARD, Harvey, B.; 11008 Lamplighter Lane, Potomac, MD 20854 (US). JACOBSON, Kenneth, A.; 11606 Fulham Street, Silver Spring, MD 20903 (US).  <b>(74) Agents:</b> KILYK, John, Jr. et al.; Leydig, Voit & Mayer, Ltd., Suite 4900, Two Prudential Plaza, 180 North Stetson, Chicago, IL 60601-6780 (US).		<b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>  <b>(88) Date of publication of the international search report:</b> <b>17 October 1996 (17.10.96)</b>	
<b>(54) Title:</b> A METHOD OF IDENTIFYING CFTR-BINDING COMPOUNDS USEFUL FOR ACTIVATING CHLORIDE CONDUCTANCE IN ANIMAL CELLS			
<b>(57) Abstract</b> <p>The present invention provides a method of identifying CFTR-binding compounds for treating cells having a reduced apical Cl<sup>-</sup> conductance, such as cystic fibrosis cells. This identification method involves the use of polypeptide I<sup>α</sup>, which constitutes a portion of the CFTR protein. The present invention also provides a method of treating CF cells by contacting cells having a reduced apical Cl<sup>-</sup> conductance with a therapeutically effective quantity of a compound selected by the present inventive identification method. Preferred compounds for such treatment have little or no affinity for adenosine cell receptors. The present invention provides novel compounds useful in practicing the present inventive method, as well as pharmaceutical compositions containing such compounds.</p>			

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## INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 95/14345A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C07K14/47 C07D473/04 A61K38/17 A61K31/52

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 C07K C07D A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SCIENCE, 1 February 1991, pages 555-557, XP000565685 THOMAS, P.J. ET AL.: "Cystic fibrosis transmembrane conductance regulator: Nucleotide binding to a synthetic peptide" * whole disclosure *	1-8,21
A	PROC. NATL. ACAD. SCI. USA, vol. 89, March 1992, pages 1539-1543, XP000565693 ARISPE, N. ET AL.: "Intrinsic anion channel activity of the recombinant first nucleotide binding fold domain of the cystic fibrosis transmembrane regulator protein" * whole disclosure *	1-8,21

 Further documents are listed in the continuation of box C. Patent family members are listed in annex.

## \* Special categories of cited documents :

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Date of the actual completion of the international search

11 July 1996

Date of mailing of the international search report

02.09.96

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Authorized officer

Hermann, R

## INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 95/14345

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,94 25605 (UNIVERSITY OF IOWA RESEARCH FOUNDATION) 10 November 1994 ---	
X	PROC. NATL. ACAD. SCI. USA, vol. 89, June 1992, pages 5562-5566, XP000565692 EIDELMAN, O. ET AL.: "A1 adenosine-receptor antagonists activate chloride efflux from cystic fibrosis cells" * fig. 2; abstract * ---	9-11, 14-16
X	EP,A,0 374 808 (BOEHRINGER INGELHEIM) 27 June 1990 * claim 1; page 5, line 50; page 22, compounds 25-30 * ---	9-20
X	WO,A,94 03456 (BOEHRINGER INGELHEIM) 17 February 1994 * claims; page 19, 3rd paragraph * ---	9-20
A	BIOCHEMICAL PHARMACOLOGY, vol. 39, no. 12, 1990, pages 1897-1904, XP000574612 BRACKETT, L.E. ET AL.: "Activities of caffeine, theophylline, and enprofylline analogs as tracheal relaxants" * tab. 1 * ---	9-20
P,X	BIOCHEMISTRY, vol. 34, no. 28, 18 July 1995, pages 9088-9094, XP000565691 JACOBSON, K.A. ET AL.: "Stimulation by alkylxanthines of chloride efflux in CFPAC-1 cells does not involve A1 Adenosine receptors" * whole disclosure * -----	9-20

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/14345

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:
  
2.  Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3.  Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

- claims 1- 8,21: CFTR-derived peptides, and DNA encoding them
- claims 9-20 : Xanthin derivatives and their use

1.  As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
  
2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
  
3.  As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4.  No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

The additional search fees were accompanied by the applicant's protest

No protest accompanied the payment of additional search fees.

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

Inten nal Application No	
PCT/US 95/14345	

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9425605	10-11-94	JP-A- 6062866 EP-A- 0699759		08-03-94 06-03-96
EP-A-0374808	27-06-90	DE-A- 3843117 AT-T- 136897 AU-B- 637990 AU-B- 4707289 CA-A- 2006387 DE-U- 8817122 DE-D- 58909657 FI-B- 96513 IL-A- 92829 NO-C- 173502 NZ-A- 231901 PL-B- 162877 PT-B- 92658 US-A- 5532368 US-A- 5175291		28-06-90 15-05-96 17-06-93 28-06-90 22-06-90 04-02-93 23-05-96 29-03-96 31-12-95 22-12-93 23-12-93 31-01-94 12-09-95 02-07-96 29-12-92
WO-A-9403456	17-02-94	AU-B- 4707193 BG-A- 99489 CA-A- 2140883 CN-A- 1086818 CZ-A- 9500348 DE-A- 4325254 EP-A- 0654033 FI-A- 950542 HU-A- 65734 JP-T- 8500344 NO-A- 950497 PL-A- 307397 SK-A- 18595 ZA-A- 9305762		03-03-94 31-01-96 17-02-94 18-05-94 18-10-95 17-02-94 24-05-95 08-02-95 28-07-94 16-01-96 07-04-95 15-05-95 11-07-95 16-06-94